

Predicting the Unpredictable: the Changing Epidemiology of *Dictyocaulus viviparus* in Great Britain.

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Abstract

Disease caused by the bovine lungworm, *Dictyocaulus viviparus*, is a cause of significant morbidity and mortality in dairy herds across the world. Subclinical infections are associated with reduced milk production in dairy cattle and growth rate deviations in beef calves whereas clinical outbreaks can be unpredictable and expensive.

Current estimates for lungworm prevalence across Great Britain are unknown but have risen significantly since the 1970s. This has been associated with more cases in adult cattle and the disease emerging into more northern regions of England and Scotland. The objectives of this thesis were to quantify and understand changes in lungworm epidemiology which have occurred over the past 40 years, understand the relative role that farm management factors and climate (change) have had on the changing prevalence of the disease and to predict future changes which may occur under climate change conditions.

Chapter 2 investigates the spatiotemporal distribution of lungworm cases reported to the Veterinary Investigation and Diagnosis Analysis (VIDA) database from 1974 – 2014. There has been a significant increase in the diagnostic rate of lungworm across Great Britain from 0.97 cases per 1,000 submissions in 1980 to 4.04 cases per 1,000 submissions in 2014, with a dramatically increased rate in Scotland. Moreover, the number of cases in adult cattle has increased since the 1980s. Chapter 3 describes the novel use of an existing *D.viviparus* ELISA in dairy herds, with an improved test sensitivity of 66.7% and specificity of 95.5% under field conditions. Chapter 4 subsequently made use of this test in a cross - sectional survey of UK dairy farmers, which improved our understanding of farm management practices associated with an increased risk of *D.viviparus* presence. Chapter 5 quantifies the influence of climatic conditions, building a mathematical model, which predicts the development, survival and migration of infective *D.viviparus* larvae on pasture. It was validated using a longitudinal study and showed that pasture infectivity occurred 46 days prior to a peak in antibody response (95% confidence intervals 38 – 52 days). The model demonstrated that the climate was conducive to increased *D.viviparus* transmission in Scotland from 1995 onwards. Under future climate change predictions, by 2055, the majority of England is predicted to have a climate not conducive to *D.viviparus* transmission. Exceptions to this are in Scotland and the southwest of England, which are predicted to remain hotspots for the disease until at least 2095.

In the past, researchers have largely attempted to explain the changes observed in lungworm epidemiology from farm management factors. However, the findings presented here suggest that climate (change) can account for most of the changes observed and may have already had a significant impact on the epidemiology of *D.viviparus* across Great Britain. Future global changes in livestock farming will continue to threaten the stability of the disease landscape. Mathematical models such as the one described here, can forecast heightened disease risk periods and will be useful in the design of sustainable control measures for lungworm disease.

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This thesis is dedicated to

my Gran, Dot

Laughing until the end.

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Chapter 1: Introduction

1.1 Food security and the role of helminth infections in farmed ruminants

1.1.1 Current challenges in Veterinary parasitology

“Food security exists when all people, at all times, have physical and economic access to sufficient safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life” (FAO, 1996). In order to meet the needs of an expanding population, there is a target for global food production to increase by 70% by 2050 (FAO, 2009). Livestock farmers need to balance the pressures of an ever unstable economic climate and decreasing profit margins with increased consumer concerns about residues in food (Kang et al., 2017), impact that farming products may have on the environment (Cooke et al., 2017) and greenhouse gas emissions (Özkan et al., 2016). Helminth infections are a major threat to the livestock industry by causing production losses and necessitating the need for chemical control measures, which negatively affect the environment. For example, liver fluke infection in cattle is estimated to increase greenhouse gas emissions by 10% for every affected cow (Williams et al., 2015). Conversely, adequate anthelmintic control practices can reduce greenhouse gas emissions and help to reduce the carbon footprint of livestock farming (Kenyon et al., 2013).

Gastrointestinal nematode infections are present in all grazing livestock (Morgan et al., 2013). Parasitic gastroenteritis mainly comprises *Ostertagia ostertagi* and *Cooperia oncophora* in European cattle; *Teladorsagia circumcincta*, *Haemonchus contortus*, *Trichostrongylus* spp. and *Nematodirus* spp. in European sheep and goats and *Ascaris suum*,

Trichuris suis and *Oesophagostomum dentatum* in domestic pigs (Charlier et al., 2018). Significant lungworms of ruminants include *Dictyocaulus viviparus* in cattle and *Dictyocaulus filaria*, *Protostrongylus rufescens* and *Muellerius capillaris* in sheep and goats whereas the trematode class of parasites, most notably *Fasciola hepatica*, the common liver fluke and *Paramphistomum* spp., the rumen fluke, cause significant pathology in cattle and sheep.

Helminth infections are a major economic constraint on efficient livestock production (Charlier et al., 2014b). Production losses such as reduced milk yield, growth rate deviations, reduced carcass quality and infertility are seen in both subclinical and clinical nematode infections (Charlier et al., 2014b). In a series of similar studies, bulk tank *Ostertagia ostertagi*, *Fasciola hepatica* and *D.viviparus* antibody titre increasing from the 25th to 75th percentile were associated with milk production losses of 1.1kg per cow per day (Charlier et al., 2005a), 0.7kg per cow per day (Charlier et al., 2007) and 0.5kg per cow per day (Charlier et al., 2016a) respectively. Reduced feed intake is the major cause of subclinical production losses (Forbes et al., 2009) with, for example, anthelmintic – treated dairy cows grazing 47 minutes longer per day than controls on nematode infected pasture leading to a significant increase in milk yield (Forbes et al., 2004). Traditional estimates of the cost of parasitic nematode infections are likely to be underestimates. Recently, the concept of ECONOHEALTH was introduced. In this framework, the major diseases affecting livestock are considered simultaneously and within the whole farm social and economic context (Charlier et al., 2015). For example, altered grazing season lengths due to heightened nematode risk periods are likely to have an impact on the whole farm economics (van der Voort et al., 2017, 2013) and are often neglected in calculations for the cost of nematode infections.

A significant barrier to effective future parasite control is that farm-based recommendations, spatial prevalence and temporal risk periods may change simultaneously under future climate change conditions. It is predicted that warmer autumn and spring months will lead to a prolonged grazing season across most European countries, which would alter the transmission opportunities of parasites on pasture (Phelan et al., 2016). Hot, dry summers could lead to a biphasic peak in infection pressure of *Haemonchus contortus* on pasture (Rose et al., 2016) whilst there is already evidence that climate change is altering the epidemiology of parasitic gastroenteritis in sheep (van Dijk et al., 2008). The challenges posed by climate change represent a dynamic system, where current advice will need to be reassessed and altered to reflect a changing environment. The free living larval stages retain the ability to adapt to changing climatic conditions (van Dijk and Morgan, 2008) but the degree to which each parasite species will evolve is unknown and will vary between parasites. There is an increasing need to i) predict the effect that climate change may have on parasite control and epidemiology and ii) set guidelines and timescales by which to reflect upon any epidemiological changes and evolution of the parasite.

The availability of cheap anthelmintics and relatively expensive veterinary expertise has led to an indiscriminate use of anthelmintics such that the escalating spread of anthelmintic resistance is considered the single biggest threat to sustainable nematode control (Charlier et al., 2018). Anthelmintic resistance is a major problem in gastrointestinal nematodes of sheep and is an emerging problem in cattle. To date, the majority of cases concerning anthelmintic resistance in nematodes in cattle in the United Kingdom involve ivermectin – resistant *Cooperia* spp. (Stafford and Coles, 1999) although it has been reported in several additional parasitic species worldwide (Sutherland and Leathwick, 2011). To date, resistance of *D. viviparus* to any of the major anthelmintic classes has not been reported.

1.1.2 Attempts to overcome the negative consequences of global change

Unlike many of the zoonotic or endemic bacterial or viral diseases of livestock, parasitic diseases have failed to gather political interest from national or international government policy makers (Charlier et al., 2018). Therefore, to help guide evidence – based, sustainable control practices, several pan – European consortiums have been established (for example, COST Action CAPARA, EU FP6 PARASOL, EU FP7 GLOWORM, EU Horizon2020 Paragone, LiHRA). This has led to the successful creation of local and national advisory bodies such as SCOPS (Sustainable Control Of Parasites in Sheep) and COWS (Control Of Worms Sustainably) in the United Kingdom (Taylor, 2012). Mathematical models are becoming increasingly utilised within parasitology consortiums to guide decision making and to predict and therefore prevent future changes. The vision for 2030 here is the “full integration of parasite population models with production and management, calibrated by real-time on-farm automated data collection” (Vercruysse et al., 2018). Although such integration would undoubtedly provide valuable data for real-time parasite disease risk, the “full” integration, including individualised on-farm model use, seems an overly optimistic target by 2030. Particularly so when considering that mathematical models have yet to be produced for many of the major parasite species, including *D. viviparus*. A more realistic target would be to develop and validate mathematical models for all of the most important parasitic species and moreover to produce a centralised model repository with a user-friendly interface. This would enable farmers and veterinarians to evaluate local disease risk based on regional climatic data. This would be a useful first-step in working towards the full integration of on-farm models.

There have been some recent novel advances in the diagnosis of helminth infections in ruminants. Next generation sequencing offers the ability to characterise and quantify the

species composition of nematode communities (Avramenko et al., 2015). The advent of pen – side technologies such as the mini-FLOTAC (Cringoli et al., 2013) and FECPAK (Techion, n.d.) offer practical, rapid advice for farmers without the need for expensive veterinary diagnostics. Precision livestock farming, where animals are monitored continuously in real-time has the potential to provide an abundance of information on an individual animal's health (Berckmans, 2017). Sensors on pasture could detect subtle meteorological changes and when combined with appropriate mathematical models, could alert a farmer to increasing risk of parasite transmission on pasture (Verschave et al., 2016). Automated systems currently exist to detect lameness or the onset of oestrus in cattle (Norton and Berckmans, 2017). Precision livestock farming tools which could detect changes in milk yield, food intake or even recognise coughing in a group of animals are likely to be highly beneficial in enhancing the diagnosis of lungworm.

Concerns regarding the emergence of anthelmintic resistance has led to an increased research interest in finding methods for controlling parasites sustainably. Targeted treatments (TT) and targeted selective treatments (TST) aim to control production losses whilst preserving a pool of untreated parasites in refugia therefore preserving anthelmintic efficacy (Charlier et al., 2014a). By 2030, it is predicted that we will enter an “new anthelmintic era”, characterised by innovative control approaches together with enhanced diagnostics meaning that only animals in need of anthelmintic treatment are targeted at the right time (Vercruysse et al., 2018). Various parasitological (e.g. faecal egg counts), production (e.g. poor weight gain or body condition score) or morbidity (e.g. serum pepsinogen levels) guidelines have been considered to apply targeted selective treatment (Vercruysse and Claerebout, 2001; Charlier *et al.*, 2014). The only paper to consider the use of targeted selective treatment for lungworm disease in dairy cattle found that the

application of eprinomectin or ivermectin was successful in reducing infection levels but failed to eradicate disease on farm despite intensive monitoring, testing and treatment of every seropositive animal in the herd (Höglund, 2006). Possible reasons for this failure include the insensitivity of the ELISA test to diagnose lungworm on farm. Similar failures were noted when attempting to eradicate lungworm on farm by the targeted treatment of animals who had grazed during the previous grazing season (Ploeger and Holzhauser, 2012). In a very highly fecund parasite species, population ‘explosions’ occur rapidly when the circumstances at pasture favour development and survival. If we are to get a hold on whether TST approaches can be adopted for lungworm, we need more accurate diagnostic tests and further work to understand when it would be necessary to treat animals.

1.1.3 Knowledge gaps in the control of helminths under global change

Despite recent advances in the field of nematodes in ruminants, many research gaps remain (Charlier et al., 2018). The ability to accurately confirm the presence or absence of a parasite on farm could feed directly into accurate spatiotemporal prevalence estimates, encourage the establishment of region specific control measures and minimise the subclinical economic impact of the infection. However, despite advances in novel diagnostic methods, many of the basic parasitological techniques for detecting the disease on farm (such as faecal analysis and antibody monitoring) are limited by poor test performance and cannot be used in this fashion. There is an urgent need for more diagnostic tests, which can detect the presence of the parasite on farm, prior to subclinical losses or clinical outbreaks being witnessed.

Although it is undoubtable that climate change will have an impact on the global prevalence of helminth infections, the degree to which farm – based management factors can counteract or confound these levels is unknown. Furthermore, the relative significance of meteorological compared to farm management factors in influencing the incidence of parasite infections under global change has received little research attention. There is a growing need to understand the complicated interactions between different factors in order to accurately predict future changes. Mathematical modelling offers the opportunity to capture this complexity. Further development of properly validated and analysed models should be encouraged.

A final major obstacle to parasite control lies in the lack of awareness and interest amongst farmers, veterinarians, researchers and policy makers on the significance of helminth infections for farmed ruminants. Helminth infections fail to attract the research interest that they deserve. None so is this more poignant than in the case of *D.viviparus* amongst dairy cattle. Despite well – documented studies on the impact that the disease has amongst the livestock community, research interest in the disease has decreased dramatically over the past few decades. Amongst the scientific community, non-gastrointestinal helminth infections are regularly excluded from scientific consortiums (for example EU FP7 GLOWORM) and review studies (see for example Charlier *et al.*, 2018). Despite not fitting within the parasitic gastroenteritis complex, infections such as liver fluke maintain a strong research interest. This is not the case for lungworm. There is a need to retain the knowledge and awareness of this disease in order to limit the potentially serious economic, environmental and welfare consequences to livestock farmers.

1.2 The impact and epidemiology of *Dictyocaulus viviparus* in farmed cattle

1.2.1 Historical account of the disease

Clinical disease caused by parasitic bronchitis has long been recognised as a significant cause of morbidity in cattle and an economic disease of Veterinary importance. Clinical descriptions of “Hoose” can be seen in textbooks from at least the early 19th Century (see for example Boardman, 1805) although it is only in the early 20th Century that identification of the disease as having a parasitic origin and therefore differentiation from other causes of acute bronchitis were commonplace. Early texts refer to the parasite as *Strongylus micrurus* and incorrectly assumed a role for earthworms as an intermediate host (Smith, 1905). The taxonomic name *D.viviparus* (Nematoda: Strongyloidea) can be seen in circulation since the 1920s (see for example Daubney, 1920).

1.2.2 Economic impact of *Dictyocaulus viviparus* infection

Aside from the welfare implications of the disease, *D.viviparus* infections are economically important to the dairy industry. Estimates of milk production losses vary between sources but patent infections have been associated with a lower average daily milk yield of 1.62 kg/cow/day in German dairy herds (May et al., 2018) whereas subclinical herds with a positive bulk tank lungworm status in August had a reduced milk yield of 1.68 kg/cow/day (Dank et al., 2015). For comparison, an increased *Ostertagia ostertagi* antibody titre in bulk tank sampling was associated with a decreased milk production in the region of 1.1 kg/cow/day in the spring and 0.9 kg/cow/day in the autumn (Charlier et al., 2005b). Clinical lungworm outbreaks are even more costly. Conservative estimates during two clinical outbreaks in the Netherlands from 2004 – 2007 caused an estimated cost ranging from

£11,029 – £17,473 (£100 - £116 / adult cow in the herd) with a significantly higher acute milk production loss of 4 kg /cow/day (Holzhauer et al., 2011). The current cost of clinical outbreaks is likely to be higher when considering not only the cost from lost income, but also additional costs due to increased animal mortality and veterinary treatment. Moreover, in beef herds there was a dose – dependent decrease in weight gain with negative herds gaining 4.5kg in weight, herds with less than 10 larvae / 30g faeces gaining 0.6 kg and herds with more than 10 larvae / 30g faeces losing 18.6kg weight over the study period (Ploeger et al., 1990c).

1.2.3 The parasite lifecycle

1.2.3.1 Preinfective stages

D.viviparus has a direct lifecycle (Figure 1-1). In contrast to the gastrointestinal nematodes, first stage larvae (L1) rather than eggs are present in the faeces. At pasture, parasite development (L1 to L3) is rapid and, if outdoor temperatures are conducive to development, is normally completed in one week (Jørgensen, 1980a). The rapid development of the preinfective larval stages is aided by the maintenance of a high number of lipid levels within the larval body such that the larvae are not thought to feed in the preinfective stage (Croll, 1973). The presence of these food granules can help to aid microscopic identification of the larvae. The free living *D.viviparus* larvae have an average length of 300 – 360µ with 20 – 24µ breadth (Daubney, 1920). The L1 and L2 larvae have the largest number of food granules, extending to within 50µ of the tip of the tail compared to L3 larvae where food granules terminate 85µ from the tip (Daubney, 1920).

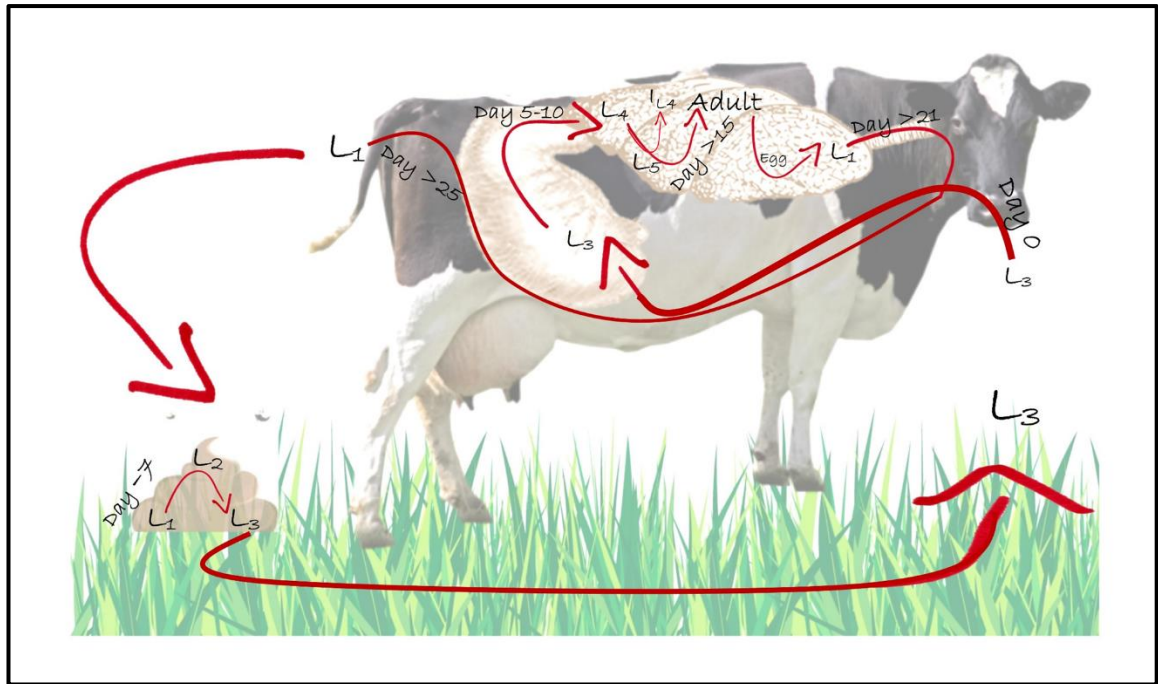


Figure 1-1 The lifecycle of *Dictyocaulus viviparus*. First stage larvae (L1) are deposited in the faeces, which develop through to the infective third stage larvae (L3) within an average of seven days. Third stage larvae (L3) are dispersed onto the pasture using the synergistic fungus, *Pilobolus* spp. and ingested L3 in the rumen migrate to the lungs and develop through to the adult stages from day 15. Larvae may undergo hypobiosis at the 4th or 5th larval stage (L₄). Adults produce eggs, which hatch in the lungs allowing L1 larvae to be coughed up by the host, swallowed and pass out in the faeces from day 25. [Source: C.McCarthy]

1.2.3.2 Infective stages

After ingestion of infective third-stage larvae (L3) with herbage, these penetrate the walls of the gastrointestinal tract and travel via the lymphatic system to the lungs where they develop through to the adult stages (Soliman, 1953). Migration within the host is stimulated by high partial pressures of carbon dioxide ($p\text{CO}_2$) in the rumen and the presence of bile, which is thought to produce specific chemoreceptors which encourage migration (Jørgensen, 1980b, 1975a). Larvae may arrest at the L4 stage and undergo hypobiosis in the host if conditions are non-conducive to further growth. This can be replicated in the laboratory by the incubation of L3 at 4°C for 6 weeks (von Samson-Himmelstjerna et al., 1999). Adult stages are present in the larger and medium bronchi (Figure 1-2) where they can clump and occlude the lumen whereas ova and larvae reside in the smaller bronchioles (Daubney, 1922). The female worms are highly fecund, producing approximately 6000-7000 L1 / female worm / day

(Kooyman et al., 2003). Eggs are coughed up by the host, swallowed, hatch and pass out as first-stage larvae (L1) in the faeces.

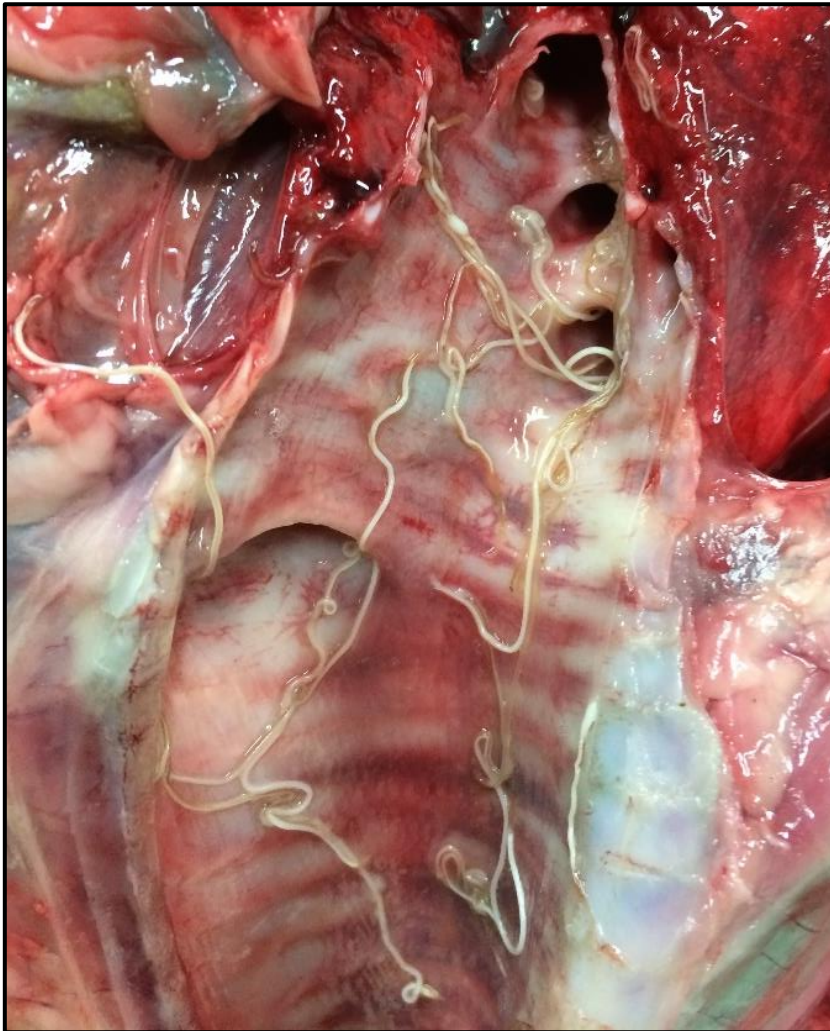


Figure 1-2 Post mortem photograph showing adult Dictyocaulus viviparus present in the larger and medium bronchi from a cow who died from clinical lungworm disease. [Photograph: B. Strugnell, Farm Post Mortems Ltd]

1.2.3.3 Effect of temperature and rainfall on lifecycle stages

Under laboratory conditions, eggs (measuring 78 – 96µ in length and 51 – 58µ in breadth) hatch almost immediately, with the transition from L1 to L2 stages occurring within 6 to 12 hours and the further development onto the L3 stage within 12 to 48 hours (Daubney, 1920). Lower temperatures delay the development of the preinfective stages with this process taking up to six days at 10°C but only three days at 22°C (Jørgensen, 1980a). However, survival of free living larvae is prolonged at low temperatures with L3 being able to withstand repeated cycles of freezing and thawing (Daubney, 1920). Lower temperatures have also been shown to delay the timing of egg hatch by up to 24 – 72 hours at 8°C (Daubney, 1920) although these conditions are unlikely to exist within natural host stages. However, the larval stages, particularly L1 and L2 are sensitive to desiccation where mortality can occur within minutes (Daubney, 1920). A longitudinal study looking at pasture infection dynamics found that despite variable temperatures, pasture contamination appeared to occur almost exclusively 1 week after faecal larval output and faecal output occurred roughly 4 weeks after pasture contamination (Jørgensen, 1980a).

1.2.3.4 Development of the synergistic fungus, *Pilobolus* spp.

A fascinating contribution to the epidemiology is delivered by *Pilobolus kleinii* and *P. crystallinus* fungae (Phycomycetes: Mucorales). While *D. viviparus* development is completed in the cowpat, the fungus grows on top of it (Figure 1-3). In the absence of *Pilobolus* spp., free – living larvae will migrate under temperature conditions within 21 – 27°C with water saturation (Daubney, 1920) and will move towards a light source (Doncaster, 1981). However, they were only found to migrate vertically by a maximum of one inch (Rose, 1956). *Pilobolus* spp. are thought to produce chemokines, which direct the usually sluggish L3 upwards and migrate to the head of the fungus. Interestingly, during laboratory

experiments, *D.viviparus* were found to be the only nematode which could reach the base of the sporangia in numbers in excess of 50 larvae per sporangia; other strongyles did not even reach the sub-sporangial swelling (Doncaster, 1981). When the spore box of the fungus discharges, the infective lungworm larvae are expelled up to 10 feet away from the faecal pat, thereby increasing the chance of ingestion by grazing cattle (Robinson, 1962). Importantly, larvae at a distance at least 100cm from the faecal pats were at the same maturation point as those within the faeces, suggesting a rapid translation onto the pasture which exceeds the rate at which the larvae would be able to actively migrate in the absence of *Pilobolus* spp. (Jørgensen, 1980a). In field experiments, calves grazing pasture containing *Pilobolus* spp. developed a 10-fold increase in faecal larval count, 7-fold increase in worm counts at necropsy and lost 0.25kg in weight compared to calves grazing pasture in the absence of *Pilobolus* spp. who gained 26kg in weight over the study period (Jørgensen et al., 1982).



Figure 1-3 Abundant growth of *Pilobolus* spp. on the surface of cattle faeces growing in the laboratory. Infective, third stage larvae (L3) migrate to the top of the mature sporangiophores. Daily *Pilobolus* spp. sporulations cause the L3 to be dispersed away from the faeces and onto the pasture. [Photograph: C. McCarthy]

1.2.4 Dictyocaulosis in non-cattle species

There is ongoing controversy regarding the taxonomy and speciation of the *Dictyocaulus* genus (Strongylida: Dictyocaulidae) (Gasser et al., 2012; Höglund et al., 2003b) with three key species parasitizing domestic livestock: *D. viviparus* in cattle (*Bos taurus*), *Dictyocaulus filaria* in lambs (*Ovis aries*) and *Dictyocaulus arnfieldi* in donkeys (*Equus africanus asinus*) (Rode and Jørgensen, 1989). Various wild ruminants have been shown to harbour *Dictyocaulus* spp. including *D. eckerti* (Gasser et al., 2012) and *D. cervi* (Pyziel et al., 2018) in red deer (*Cervus elaphus*) and *D. capreolus* in roe deer (*Capreolus capreolus*) and moose (*Alces alces*) (Höglund et al., 2003b). *D. viviparus* enjoys a wide geographical dispersion from farmed cattle in Quebec, Canada (Gupta and Gibbs, 1969) and mountain pastures in French cattle herds (Raynaud and Gruner, 1982) to wild Bison (*Bison bison*) in Montana, USA (Locker, 1953).

D. viviparus is also a frequent cause of morbidity in deer, causing high mortality in young, farmed, red deer (*Cervus elaphus*) in New Zealand (Charleston, 1980) with a prevalence as high as 84% of red deer farms (*Cervus elaphus*) and 100% of fallow deer farms (*Dama dama*) (Mason and Gladden, 1983). Prevalence levels amongst non-farmed ruminants is high with *D. viviparus* reported in 14% of moose (*Alces alces*) in Alberta, Canada (Pybus, 1990) and between 32-70% of Rocky Mountain Elk (*Cervus canadensis*) in Wyoming, USA (Bergstrom, 1975). *D. viviparus* appears host adapted to cattle and ruminants with experiments failing to infect domestic pigs (*Sus scrofa domesticus*) (Tromba and Douvres, 1957). However, the successful infection of guinea pigs (*Cavia porcellus*) (Douvres and Lucker, 1958) have enabled these animals to be used as calf models in laboratory studies (Cornwell and Jones, 1971).

Transmission amongst deer has been reported since the 1940s (Dougherty, 1945; Erickson and Highby, 1942) and during the 1970s in particular, questions were being asked as to the role of wild deer in the epidemiology of *D. viviparus* to domestic cattle. However, experimental studies failed to find a definitive link between the two species. Adult *D. viviparus* larvae taken from a dead moose (*Alces americanus*) in Northern Quebec failed to infect two experimental calves despite high numbers of larvae (6,000 and 12,000) being administered (Gupta and Gibbs, 1971). Conversely, similar studies using larvae from black tailed deer (*Odocoileus hemionus columbianus*) did successfully colonise calves, which developed clinical signs of infection 16 – 24 days post-challenge (Presidente and Knapp, 1973). However, at post-mortem these larvae had failed to reach full maturity to adult worms, therefore preventing full-cycle oviparous establishment. Therefore, the role that deer play in maintaining the infection within cattle herds is likely to be minimal.

1.2.5 Host stages and the development of immunity

The classical clinical presentation of lungworm is described in a textbook for the breeding farmer, cowkeeper and grazier from 1822 as:

“This disorder, known by the names of Fog Sickness, and Rising of the Lights....is known by great difficulty of breathing, attended with a cough, or hoose; the animal opens her mouth wide, the tongue is thrust out, the nose and mouth run out a ropy slime, the eyes appear dull and heavy...and as the disease proceeds, the animal becomes very restless and unmanageable.” (Skellett, 1833)

D. viviparus infections can cause a reactivation of previous infectious bovine rhinotracheitis (IBR, bovine herpesvirus 1) infections (Msolla et al., 1983) which can complicate the clinical signs, often leading to profuse nasal discharge (Figure 1-4). One of the first pathological lesions occurs in the mesenteric lymph nodes where small necrotic lesions can be seen from day three post-ingestion (Jarrett and Sharp, 1963). Gross pathological changes are consistent with atelectasis, vesicular emphysema, consolidation of apical and cardiac lobes and the presence of a mucopurulent exudate (Daubney, 1922; Parker, 1957) (Figure 1-5). Microscopically, pathology is associated with bronchi plugs, mixed infiltration with necrotic foci, secondary bacterial invasion and the formation of lymphoreticular broncho – occlusive lesions from day 21-35 (Daubney, 1922; Jarrett and Sharp, 1963).



Figure 1-4 Dairy cow showing the profuse nasal discharge from an infectious bovine rhinotracheitis (IBR) infection. Note the tongue extension and open – mouthed breathing often seen with lungworm disease. [Photograph: J. van Dijk]

Infected cattle will mount a complement fixing and gamma globulin immune response against *D. viviparus* (Weber, 1957) with a strong negative correlation between IgE levels and lungworm burden at necropsy ($r=-0.88$ to 1) (Kooyman et al., 2002). The authors argue that high IgE levels may indicate a bias towards a Th2 weighted immune response which may be the protective factor rather than IgE itself. Faecal patency is first detected from 18-22 days post infection (DPI) with seroconversion occurring later at 30-44 DPI (Schnieder, Bellmer and Tenter, 1993). Antibodies can be detected in either milk or serum sampling and persist until 112-138 DPI in milk and 126-143 DPI in serum (Fiedor et al., 2009). There is no cross-immunity between either *Ostertagia*, *Cooperia* or *Dictyocaulus* in calves (Kloosterman and Frankena, 1988).

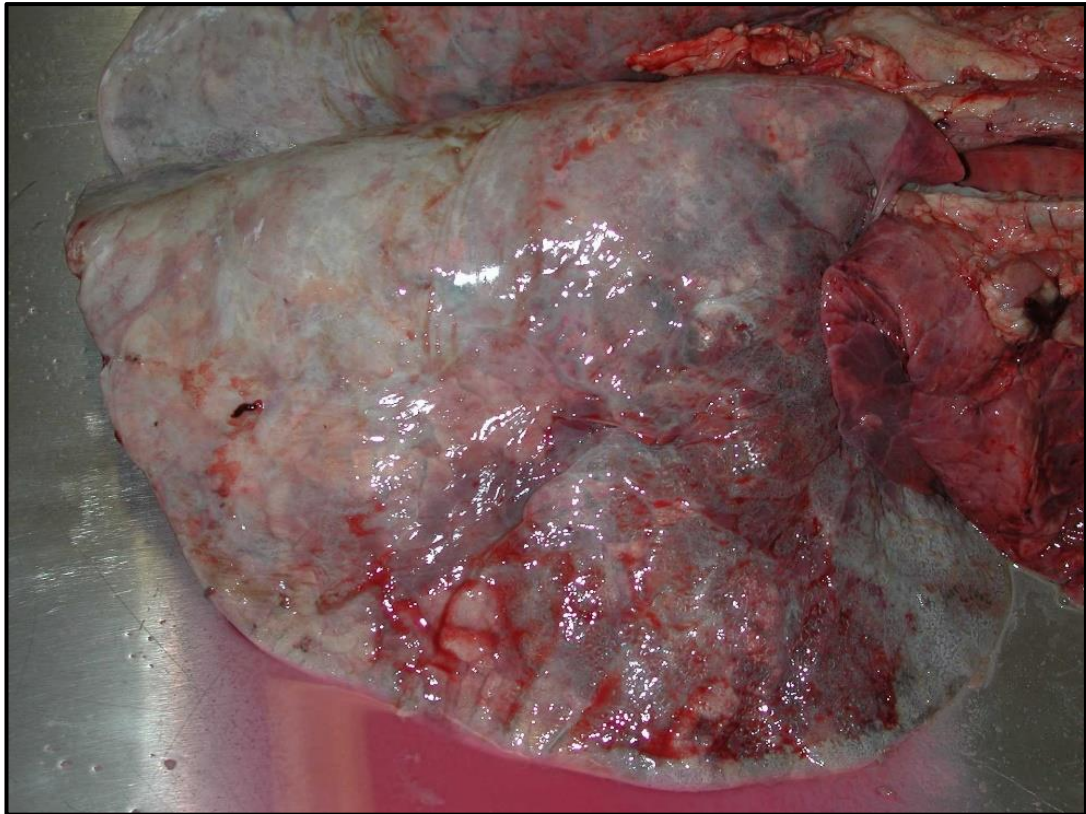


Figure 1-5 Post mortem photograph showing lungs from a cow who had died from clinical lungworm disease. The very extensive (subpleural) emphysema, associated with partial obstruction of the bronchi with parasites and exudate / transudate is evident. This emphysema presents a severe welfare problem. [Photograph: J. van Dijk]

At first infection, faecal patency lasts for around 3 months in the absence of treatment (Michel and Mackenzie, 1965). Immune calves are both able to eliminate the majority of their parasite burden by day 10-30 post infection, and prevent the further establishment of parasites after the 11th day (Michel and Mackenzie, 1965). In comparison to naïve cattle, the lungworm burden which established in immune cattle consisted of 70% fewer parasites which are 25-35% shorter in length, have a 38-77% reduction in parasite fecundity (fecundity strongly correlated with parasite length) and have a 10-15% higher proportion of female parasites (Kooyman et al., 2003). Therefore although resistant calves display an increased respiratory rate by day 7-10 post re – infection (Michel and Mackenzie, 1965) these infections are unlikely to be patent.

The immune response is targeted against two stages of the parasitic lifecycle. The immunity which targets larvae coming into the lungs lasts less than six months whereas the immunity targeted against adult parasites in the bronchae lasts up to two years (Michel and Mackenzie, 1965). Therefore, the duration between first and subsequent reinfections is important in disease pathogenesis. If reinfections occur between six months and two years after primary infection, cattle will have lost the immunity which prevents incoming parasites from establishing, but will have maintained the immunity that kills parasites within the lungs. The eosinophil response in naïve animals is evident from 10 DPI and peaks by 2-2.5 weeks (Michel and Mackenzie, 1965). However, if the immunity has lapsed, the eosinophilic response to the sudden death of a large number of incoming parasites can lead to an abrupt, excessive hypersensitivity reaction which can lead to sudden death of the cattle in a process known as “reinfection syndrome” (Michel and Mackenzie, 1965).

There is no real evidence for an age-related immunity to lungworm but instead the immune response depends on previous lungworm exposure and individual host factors. In many cattle, low infection doses are sufficient to prime the immune response against later reinfections. Pre-priming with as few as 30 larvae produced, on average, 70.3% reduction in the number of parasites at necropsy when challenged with 2000L3 at day 35 (mean lungworm burden 145 compared to 489 in controls) (Kooyman et al., 2003). However, one calf had an initial dose of 960 larvae but produced very low IgE titres and was not protected against rechallenge (Kooyman et al., 2002). More recent studies have found the antibody response to be dose-independent at infection doses of 25 larvae and above (Strube et al., 2017). The host factors driving this difference in immune response are currently unknown and deserves further research attention.

1.2.6 Transmission on pasture

Pasture contamination typically consists of three waves of infection across the grazing season, beginning 2 – 4 weeks post-turnout, with cyclical increases in pasture contamination levels occurring every 4 weeks until immunity has developed (Eysker et al., 1993; Jørgensen, 1980a). The wave in which clinical disease is observed depends on a balance between the level of immunity within the herd and the relative pasture infectivity but is likely to occur within the second (Eysker et al., 1996b; Michel, 1969) or third (Eysker et al., 1995b) wave.

There is evidence to suggest that larvae may survive overwinter in low numbers on pasture (Hertzberg et al., 1996; Jørgensen, 1980a; Oakley, 1982) but the majority of the year – year maintenance of the parasite within herds is largely dependent upon the parasites undergoing hypobiosis within adult cattle (Eysker et al., 1994b). An interesting study looking at the causes of lungworm outbreaks on Dutch farms found that 20 out of 25 cases were due to contamination by carrier animals in spring (Saatkamp et al., 1994). However, the same study found that in four cases, an outbreak was caused by the parasites surviving overwinter on the pasture. Abattoir studies from Canada found that during the winter 0.19% of cattle had lungworms present in their lungs (Gupta and Gibbs, 1969). This may reflect the number of carrier animals within the herd.

Earthworms have been found to transmit *Ostertagia ostertagi* from faeces to soil (Grønvold, 1979). *D.viviparus* larvae have been found within earthworms and homogenised, infected earthworms can infect six-month-old calves (Oakley, 1981). Interestingly, a longitudinal study looking at pasture plucks from 1976 to 1979 found that pasture infectivity decreases after

cattle are removed but then increase before cattle resume grazing on the pasture (Oakley, 1982). The authors argue that this could be due to translation by earthworms. The exact conditions needed to encourage this process are unknown. Although this may be a mechanism to enhance the overwinter survival of larvae on pasture, the effect that this process has on the infectivity of the larvae are unknown.

Due to the need for pasture infectivity to build up over the grazing season, lungworm disease shows a strong seasonal trend. The incidence of lungworm in lung samples from an abattoir in Quebec, Canada was highest in the Autumn (5.63% lungs), followed by Summer (2.83%), Spring (1.12%) and Winter (0.19%) (Gupta and Gibbs, 1969). Similarly, a 1-year longitudinal study examining monthly individual milk samples from 519 cows in Germany, found August to reveal the highest prevalence (10.23% animals) with the lowest in March (0.85% animals) (Schunn et al., 2012). Interestingly, an Irish study found a contrasting seasonal pattern. Bloemhoff (2015) showed that November (54.8% farms, 95% confidence intervals 48.8 - 60.8) and March (17.4%, 95% confidence intervals 12.9 - 21.9) showed the highest farm prevalence values with a decrease in June (8.1% 95% confidence intervals 4.9 - 11.4) and August (2.2% 95% confidence intervals 0.5 - 4.0). This study analysed bulk milk samples from 290 farms on a quarterly basis and considering milk ELISA values can remain positive for 79 – 107 days (Fiedor et al., 2009), the apparently higher seroprevalence in November may reflect infections occurring several months previously.

1.2.7 Current diagnostic options for *Dictyocaulus viviparus* in dairy herds

Diagnosis of lungworm disease can be challenging even during a clinical outbreak. Clinical signs can be seen by 13 days post-infection in calf studies when 10,000 larvae were administered to 3 month-old calves (Parker, 1957). Increased respiratory rates can be

expected from the second (Michel and Mackenzie, 1965) to third (Boon et al., 1984) week post infection which is earlier than faecal patency or seroconversion. Furthermore, clinical signs are an inaccurate tool for diagnosis with farmers failing to notice clinical signs in 36.2% of seropositive herds whereas only 50.8% of herds were seropositive when farmers suspected they had developed clinical signs (Schnieder, Bellmer and Tenter, 1993).

Faecal patency begins from day 23 – 28 post infection (Fiedor et al., 2009). The Baermann test is sensitive enough to detect 1 patent female worm from 4.5 – 5 weeks post infection (Eysker, 1997). However, false negatives can occur prior to faecal patency or if larvae die within faeces prior to Baermannization. Recovery rates are reduced by faecal storage so that only 80% of larvae will be recovered after storage at 4°C for 24 hours, reducing to 40% at 20°C and 20% after 48 hours at 20°C (Rode and Jørgensen, 1989).

A novel *D.viviparus* genus-specific 17kDa antigen was discovered by de Leeuw and Cornelissen (1991), isolated and characterised as a pure recombinant protein Dv 3-14 by Schnieder (1992a) and found to be a major sperm protein of *D.viviparus* by Schnieder (1993b). Bioinformatic studies have found *msp* transcription in *D.viviparus* occurs primarily in hypobiotic L5 and adult male parasitic stages (Christina Strube et al., 2009). An enzyme-linked immunosorbent assay (ELISA) using MSP as an antigen was developed for use in serum (Cornelissen et al., 1997; von Holtum et al., 2008). This was adapted for use in milk samples and found to initially become positive from 30 – 32 days post infection and persist until days 126 – 143 post-infection (Fiedor et al., 2009). More recent studies have found that the peak in antibody response during a reinfection may be as late as 36 – 46 days after peak pasture infectivity (Strube et al., 2017). In serum samples, the test performs favourably, with a sensitivity reported to be as high as 97.7% (95% confidence intervals 91.9 – 99.7%) and

specificity at 98.1% (95% confidence intervals 95.3 – 99.5%) from 21-28 days post-infection, with no cross-reaction with either *Ostertagia ostertagi* or *Cooperia oncophora* (Goździk et al., 2012).

Herd-level diagnosis of the disease is more complicated. Bulk tank milk (BTM) testing using the MSP ELISA shows inconsistent results depending on month of testing and within-herd disease prevalence. Some studies report a sensitivity and specificity as high as 100% and 97.3% respectively (Schunn et al., 2012), whilst other studies report a much lower test performance of 53.3% and 88.2% (Ploeger et al., 2012). Estimates as low as 50% and 99% have been reported in August (Charlier et al., 2016a). Original development of the BTM ELISA relied on a within-herd prevalence of 20% (Fiedor et al., 2009) although field studies suggest that at least 30.7% of the milking herd needed to be seropositive before the BTM became positive (Ploeger et al., 2012). The dilution of milk antibodies within the large vat of milk may be one of the reasons behind the low test performance of the BTM ELISA. This may be overcome by testing individual, at-risk animals within the herd. An interesting study found that in herds showing clinical signs of lungworm disease, 9 heifers should be tested by faecal Baermann analysis or 6 heifers tested through serum or milk sampling in order to detect at least 1 positive animal (Ploeger et al., 2012). However, this may be cost prohibitive for regular routine testing.

Other diagnostic options have failed to gather the commercial interest that they perhaps deserve. An immunoblot based on a rapid dipstick immunoassay has been found to have a sensitivity and specificity which both exceed 99% and could make a useful pen-side tool providing results within 90 minutes with no laboratory processing and no interference from either the lungworm vaccine or gastrointestinal nematodes (Schnieder, 1993b).

Bronchoalveolar lavage offers the opportunity to isolate either *D. viviparus* larvae or monitor pulmonary eosinophilia, which, when used in combination, offers a sensitivity of 91.4% (95% confidence intervals 80.7 - 97.4%) and specificity of 85.2% (95% confidence intervals 68.8 - 99.4%) (Lurier et al., 2018).

1.2.8 Farm management risk factors for lungworm disease

There have been various studies looking at management factors which can alter the risk of lungworm disease on dairy farms. Cattle entering the farm has been hypothesised to increase the risk of introduction of the disease into a susceptible herd (David, 1999). In a Belgium study, farms which frequently purchased cattle were shown to have a higher bulk tank antibody titre than herds with less frequent purchasing (0.07 higher ODR) (Charlier et al., 2016a). The number of cattle in the herd has variably been found to either not be significant (Schnieder et al., 1993) or significant but to a small effect size (bulk tank ODR increase of 0.0004) (Charlier et al., 2016a). Stocking density at pasture was found to lead to higher rates of infection in one paddock (Oakley, 1982), which the authors argue could be because it encourages closer grazing near faeces. However, this is contradicted by a cross – sectional study in Germany which found no effect of stocking density on the seropositivity of first year grazing cattle (Schnieder et al., 1993).

D. viviparus larval counts increase on pasture over the grazing season from low levels at turnout to a high infection pressure in the autumn (Eysker et al., 1993; Jørgensen, 1980a). Therefore, a longer grazing season could be predicted to increase the risk of clinical disease. Turnout dates before the 15th May or a grazing length exceeding 150 days was associated with significantly more seropositive herds (Schnieder et al., 1993). However, in Ireland, early housing (classed as September or October rather than November or December), was

associated with an increased risk that the bulk tank milk sample would be positive (odds ratio 1.51) (Bloemhoff et al., 2014). This may however, represent a behavioural response with farmers opting to house cattle early if they had previously observed lungworm outbreaks in the Autumn (Ploeger, 2015). However, it has been proven that *Pilobolus* spp. can survive and transmit *D.viviparus* larvae within the cattle shed (Grønvold and Jørgensen, 1987a, 1987b) and so it should not be assumed that the risk of lungworm disease is entirely confined to periods at pasture. Case studies of clinical lungworm infections in housed 6 – 8 month old calves with minimal access to pasture suggests that transmission is still possible in housed cattle (Crawshaw and Smith, 2003).

Mowing the pasture prior to cattle grazing has been shown to reduce the risk of lungworm disease (Charlier et al., 2016a; Schnieder et al., 1993). However, this is contradicted by earlier studies which suggest that harrowing transposes larvae from faeces to herbage so that larval counts increase by three fold in three days (Baxter et al., 1959). Faecal larval counts are significantly reduced if contaminated pastures are rested for six weeks (Eysker et al., 1992). Due to the high risk of transmission from adult carrier animals (Saatkamp et al., 1994), mixed grazing of cattle of different ages or without resting the pasture for at least six weeks could increase the risk of parasite transmission. Yearling cattle that grazed pasture without older cattle were significantly less likely to be seropositive (Schnieder et al., 1993). However, it should be remembered that the immune response will wane if not re-challenged (Michel and Mackenzie, 1965). A degree of infection should be encouraged in young animals to activate and maintain an adequate immune response.

It has previously been suggested that spreading slurry can increase the farm to farm transmission of nematode larvae on pasture (Persson, 1974). Although *Cooperia oncophora*

eggs survived 2 to 5 days and *Eimeria* spp. oocysts survived for more than 50 days in slurry temperatures of 35°C, *D.viviparus* larvae didn't survive for more than one week (exact timing could not be deduced but assumed inactivated within a few days) (Olsen and Nansen, 1987). Therefore, transmission via slurry is not thought to be a significant risk factor for lungworm disease.

There are significant positive correlations between *D.viviparus* antibody titres and serology for either *Ostertagia ostertagi* (Frey et al., 2018; Ploeger et al., 1990a), *Cooperia oncophora* (Ploeger, Kloosterman, Bargeman, et al., 1990) or *Fasciola hepatica* (Frey et al., 2018). This suggests that either a) risk factors are shared between several different species of parasitic diseases of ruminants; or b) that parasitic infections in ruminants alter the immune response such that co-infections become increasingly likely. The presence of lentic water bodies and the proportion of grassed areas were both found to be significant risk factors for both *D.viviparus* (Schunn et al., 2013) and *F.hepatica* (Kuerpick et al., 2013) in Germany. However, it has also been found that infections with *Fasciola hepatica* will reduce the magnitude of the single intradermal comparative cervical tuberculin test to detect bovine tuberculosis (BTB) (Claridge et al., 2012). Furthermore, *F.hepatica* coinfections will reduce the specific Th1 immune response targeted against *Mycobacterium bovis* (Garza-Cuartero et al., 2016). No studies have currently been performed which directly assess how the immune response changes under coinfections with *D.viviparus* and this should be a future research target.

1.2.9 Meteorological risk factors for lungworm disease

Significant geographical clusters were found for *O.ostertagi*, *D.viviparus* and *F.hepatica* in Swiss dairy herds (Frey et al., 2018). This could relate to similarities between regions in farm management practices that increase the risk of a specific infection. For example, in Belgium,

there were significant interactions between region and mowing of pastures or purchase of animals (Charlier et al., 2016a). However, these clusters could also suggest that climatic variables influence the risk of parasitic diseases. Meteorological conditions and the presence of *Pilobolus* spp. explained 60% of the variation in L3 recovery in natural dung pats (Somers and Grainger, 1988).

Significantly more herds were seropositive in areas of Germany experiencing more than 50 days where the mean daily temperature exceeded 15°C between May and September, with the temperature between May and July appearing to be particularly critical (Schnieder et al., 1993). The development, mortality and migration of the free – living larval stages is dependent upon local temperature and moisture conditions (see for example, Jørgensen, 1980b). Drier weather during the autumn has been found to dry the surface of faecal pats, enabling larvae to survive over the winter, whereas wetter autumn and winter months cause the faecal pats to disintegrate and therefore leads to rapid mortality of the larvae (Oakley, 1982). In a study from Costa Rica, rainfall and maximum temperatures were significantly associated with faecal larval counts in a dairy farm but not in a beef farm (Jiménez et al., 2007). Whether this implies intrinsic differences between dairy and beef herds or differences in climatic conditions at two separate locations is unknown. Meteorological variables did not maintain significance during multivariable linear regression modelling of bulk tank antibody titres in Germany (Schunn et al., 2013). However, it is hard for multivariable statistical models to capture the complexity of variations in temperature and rainfall, which can occur over a grazing season and contribute towards waves of larval populations developing on the pasture. There is a need for more sophisticated mathematical models to be developed which can track the infection pressure as it develops over the grazing season. A pan-European study found interesting differences between countries in their management and climate which led

to different levels of risk for *Ostertagia ostertagi* (Bennema et al., 2010). Mathematical models, which can quantify differences in climate – driven risk, could help tailor region specific advice and disease control.

1.2.10 Controlling the risk of outbreaks on farm

Attempts to eradicate lungworm from farms by a single mass treatment of all grazing animals has proven unsuccessful in the medium to long term future (Ploeger and Holzhauer, 2012). Due to the constant threat of recirculation of the parasite, the optimum lungworm control strategy depends upon establishing a solid level of immunity amongst all grazing cattle. However, there is a fine balance between allowing sufficient low-level transmission of the parasite to encourage the establishment of immunity but prevent an overwhelming infection pressure that can overwhelm the developing immunity or lead to subclinical production losses.

1.2.10.1 Vaccination

There are several reasons why vaccination against *D.viviparus* should be a theoretical possibility, beyond that for other parasitic infections. Firstly, the lifecycle of *D.viviparus* has important parenteral stages involving migration through the mesenteric lymph nodes (Soliman, 1953) thereby evoking a strong host immune response. Secondly, the endemicity of the parasite and the cycling of larval generations on the pasture means the hosts are continuously re-exposed to the parasite, helping to maintain the immunity, unlike the comparative infrequency of other lungworms such as *Angiostrongylus vasorum* in dogs. The 1960s saw a plethora of research concentrating on the production of a safe and effective vaccine. Initial trials using whole worm antigens injected intramuscularly failed to produce a

strong immunity in challenge infections (Jarrett et al., 1960a). However, further studies, irradiating the larvae with between 20,000 - 40,000 roentgens showed promise in preventing severe clinical disease after challenge infections (Jarrett et al., 1960b).

However, larvae killed with 60,000 roentgens of radiation failed to provoke an adequate immunity in calves (Jarrett et al., 1960b) suggesting that it is the migration or presence of living larvae in the host which created immunity rather than the dead antigens alone. The irradiated vaccine larvae cause some degree of host pathology, leading to early lymphoreticular broncho – occlusive lesions (Jarrett and Sharp, 1963). Irradiated larvae will successfully migrate to the lungs but are more efficiently cleared by the host leading to fewer numbers in the lungs (203 irradiated larvae at day 11 post-infection compared to 608 non-irradiated larvae at day 13 post-infection) and a swifter removal from the lungs (zero larvae present at 35 days post-infection compared to 231 non-irradiated larvae still present at 35 days post-infection) (Jarrett and Sharp, 1963). There was also a shift in the gender ratio of the accumulated pulmonary adult parasites between irradiated and non – irradiated infections, with an average male : female ratio of 9:1 compared to 1:1 respectively (Jarrett and Sharp, 1963). Calves in lungworm – vaccinated herds were 10.5 kg heavier than those in non – vaccinated herds (Ploeger et al., 1990c) and clinical signs were observed in only 7.7% of vaccinated farms compared to 12.9% of non – vaccinated farms (Borgsteede et al., 1998).

The commercial vaccine, initially Dictol (Dictol®, Allen & Hanburys, Ware, Herts., Great Britain), with the patent bought by the pharmaceutical company Glaxo in 1987 (Glaxo, 1988) and later Huskvac (Bovilis® Huskvac, MSD Animal Health) is licenced for use in calves from eight weeks of age, reared free from lungworm infection. This poses several logistical considerations with the use of the vaccine in commercial herds. Although naturally infected

animals maintain their immunity which prevents worms establishing for two years, vaccinated animals will lose the initial resistance developed through the vaccination unless they are rechallenged within 6 months (Michel and Mackenzie, 1965). The vaccine protocol consists of vaccinating 8-week-old naïve calves and then allowing these calves to graze lungworm contaminated pasture in order to stimulate the immune response. The long duration between calving and vaccination means that the vaccination is often unfeasible to use in spring calving or beef herds. However, the vaccine has been found to have a 96.7% and 98.9% efficacy for 3 and 7 week old dairy calves respectively although this was reduced to 87% for 3 and 7 week old suckler calves under heavy experimental challenges (Benitez-Usher et al., 1976). However, the use of a potentially pathogenic vaccination in fast growing young stock with high expected growth rates could be economically unfeasible.

Despite the benefits of vaccination, vaccine use has been in decline (David, 1999; van Dijk, 2004). During the 1990s, 2.7% of herds in Germany (Schnieder et al., 1993) 20-25% in England and Wales (David, 1999), and 33.8% herds in the Netherlands (Borgsteede et al., 1998) used the vaccine. The bovine spongiform encephalitis (BSE) crisis in the United Kingdom led to a withdrawal of the vaccine from the European market in February 1996 causing farmers to look for alternative methods for disease control such as the application of long acting anthelmintics (Borgsteede et al., 1998). Furthermore, the production of living larvae for irradiation prior to vaccine use necessitates passage through live donor calves, a process that is incompatible with future ethical targets.

Given the practical and biohazard concerns around the use of irradiated larvae, active research is currently ongoing to look for alternative vaccination candidates. A recombinant vaccine would allow standardisation across vaccine batches and remove the need for animal

models in vaccine production. Paramyosin (PMY) is a muscle protein present within the pharyngeal and body wall muscles of all developmental stages of *D.viviparus* (Strube et al., 2009; Strube et al., 2015). Vaccinating with recombinant PMY (rPMY) has been shown to reduce worm burden and larval shedding by up to 54% and 57% respectively but was found to be far less effective in reducing parasite load than using the irradiated larval vaccine (Strube et al., 2015). Furthermore, large variations in host response to rPMY vaccination has so far limited its efficacy compared to control animals (Joekel et al., 2015). Asparaginyl peptidase legumain-1 (LEG-1), an enzyme in the intestinal tract and testes of *D.viviparus*, has also been tested as a recombinant vaccine candidate but initial clinical vaccination trials were disappointing (Holzhausen et al., 2018).

1.2.10.2 Anthelmintic use

1.2.10.2.1 Development of new anthelmintic treatments for *Dictyocaulus viviparus*

Cattle unfortunate enough to suffer from lungworm disease during the 19th Century, were treated as per the following contemporary account of a cow suffering from clinical disease:

“Bleeding is here...the principal remedy. She should be bled freely, and from a large orifice.

After this, the best practice is to peg the cow in the dewlap. An incision is made in the

dewlap; the skin is then to be separated from the flesh...as soon as the swelling and

inflammation of the dewlap has taken place, relief will be given to the lungs, and the

inflammation begin to lessen. In the meantime, till this takes place, the bowels should be

opened, by a moderate dose of the Epsom salts and nitre.” (Skellett, 1833)

It was not until the mid-20th Century that effective treatments started to be discovered. During the 1950s, diethylcarbamazine acid citrate, which was initially developed during the

second World War to combat filariasis in troops in the Far East, was found to be partially effective at improving the clinical signs of *D.viviparus* infection in cattle (Parker, 1957). Thiabendazole derivatives (part of the benzimidazole group or “white drenches”) were discovered in the 1970s, initially with cambendazole (Gibbs and Gupta, 1972; Hoff et al., 1970; Presidente et al., 1973). However, cambendazole appeared to show efficacy against adult but not immature stages of the lifecycle, leading to recommendations to dose with cambendazole, move to clean pasture and then re-dose 3 weeks later (Presidente et al., 1973). A new levamisole group of anthelmintics (“yellow drenches”) emerged as effective treatments in reducing the adult *D.viviparus* burden by 84 – 100% when applied dermally (Curr, 1977). The intra – ruminal devices, such as morantel tartrate (Paratect, Pfizer) bolus (Jones, 1983) showed immediate pan – European interest upon discovery (Bonazzi et al., 1983; Downey, 1983; Guldenhaupt and Burger, 1983; Jones, 1983; Prosl et al., 1983; Raynaud et al., 1983b, 1983a). Boluses of ivermectin (Egerton et al., 1986) and levamisole (Taylor et al., 1988) have also become available but are no longer available in the United Kingdom.

The macrocyclic lactone class (“clear drenches”) emerged from the late 1970s with avermectin (Egerton et al., 1979), ivermectin (Egerton et al., 1981), doramectin (Goudie et al., 1993a), abamectin (Kaplan et al., 1994), moxidectin (Eysker et al., 1996a) and eprinomectin (Shoop et al., 1996). The macrocyclic lactone class were found to have an excellent broad – spectrum efficacy against many adult and immature helminth infections in cattle and sheep. Doramectin was found to have an efficacy against *D.viviparus* of 99.9% at 14 – 18 days (Eddi et al., 1993) and 28 days (Weatherly et al., 1993) and 81.5% at 42 days post – treatment (Stromberg et al., 1999). In addition, it was found to be 99.6% effective at eliminating immature and adult stages of most nematodes including *D.viviparus* (Jones et al., 1993). In addition, the macrocyclic lactones could retain their efficacy in a wide range of

formulations. Eprinomectin pour-on had an efficacy exceeding 90% against most nematodes, including *D.viviparus*, at 28 days post-application (Cramer et al., 2000) with no significant reduction in efficacy under conditions simulating rain, wet hair coat or long coat length in cattle (Gogolewski et al., 1997). Similarly, the eprinomectin extended – release injectable formulation (Soll et al., 2013) had an efficacy against multiple gastrointestinal and lungworm infections which exceeded 96% (100% for *D.viviparus*) throughout the 120 day longitudinal study, despite continuous pasture challenge (Rehbein et al., 2013). Of particular benefit to dairy farmers is the zero milk withdrawal of the eprinomectin pour-on formulation with a reported residual activity of up to 28 days for *D.viviparus* (Merial, 2016).

1.2.10.2.2 Establishing immunity whilst under anthelmintic treatments

The advent of such effective anthelmintic treatments that can eradicate and prevent lungworm burdens by up to 95-100%, has raised questions as to whether increased treatment efficacy could compromise opportunities to develop an active immune response to the parasite. The number of lungworms at autopsy was not significantly different between calves who had been injected with 200 µg/kg doramectin and those who had been given the oral lungworm vaccine prior to a repeat challenge study (Taylor et al., 2000). The study found that immune hosts showed immunofluorescence to the larval sheath, suggesting that the larvae had penetrated the host's intestines prior to being killed by the doramectin. It is possible therefore, that more rapid anthelmintic killing could compromise the immune response further. Eprinomectin given at 18 days post infection, during the prepatent period, prevented faecal larval shedding and seroconversion in either milk or serum sampling (Fiedor et al., 2009). Partial immunity appeared to develop in calves who were treated with eprinomectin at the onset of patency at day 24. After a second challenge infection, these calves had fewer adult worms at necropsy than previously untreated controls but more than

calves who had experienced a previous infection and hadn't been treated (Höglund et al., 2003a). However, calves given eprinomectin during the patency period at day 49, ceased larval shedding without a compromise in seroconversion (Fiedor et al., 2009). A key problem surrounding these studies is that the number of larvae at pasture and ingested through cattle studies is normally much higher than the number of larvae animals cattle would encounter at pasture, particularly at turnout. Therefore, whether immunity reliably builds up when calves are treated with anthelmintics in normal farm (non-trial) circumstances remains to be seen.

The use of long acting anthelmintic treatments makes the immunity balance more complicated. Calves who had been given a fenbendazole slow release bolus (SRB) (Downey et al., 1993) or ivermectin SRB (Schnieder et al., 1996b) prior to their first grazing season had developed a partial immunity but displayed mild symptoms and a transient decrease in growth rate after a challenge infection in their second grazing season. A useful combination could be to use a SRB in combination with the oral vaccine. The use of the morantel SRB (Bonazzi et al., 1983) or the ivermectin SRB (Grimshaw et al., 1996) in combination with the oral vaccine did not interfere with the ability of the vaccine to stimulate immunity. Further enhancements to the immune response could be to use pulse release boluses (PRB) which dispense pulses of anthelmintics at set time periods, therefore allowing some development of the lifecycle within the host with subsequent immune development. A study comparing the immunity between naïve controls, previously infected controls, calves who had been treated with ivermectin or oxfendazole PRB prior to a challenge study found that oxfendazole PRB treated calves had a modified immune response with fewer larvae at necropsy (Jacobs et al., 1989).

The use of long acting anthelmintics poses ethical concerns about enhancing the development of anthelmintic resistance, particularly when at least 33.3% of herds are thought to over-apply anthelmintics (Borgsteede et al., 1998) and 30% of dairy farmers were found to use an anthelmintic which was “unsuitable for purpose” (Bloemhoff et al., 2014). Moreover, the use of the morantel SRB caused gastrointestinal faecal egg counts to rise towards the end of the grazing season as the bolus’s active life had been completed after approximately three months (Guldenhaupt and Burger, 1983). This is a particular concern for lungworm disease as morantel SRB treated animals developed more severe clinical signs and higher faecal larval counts than untreated controls towards the end of the grazing season (Downey, 1983). This has led some authors to recommended delaying the administration of the bolus to later in the season in order to provide protection in the autumn months with the highest rates of transmission on pasture (Hertzberg et al., 1996).

Rates of anthelmintic dosing show regional and temporal differences. A questionnaire study of dairy cattle farms in the Netherlands in 1996 found that 41.5% herds were given preventative anthelmintic treatments at turnout, of which 66.9% were given long – acting boluses, 36.6% herds were given treatment during the grazing season and 50.3% were given anthelmintics at housing (Borgsteede et al., 1998). An equivalent questionnaire of Irish dairy farmers in 2009 found that anthelmintics were given to dry cows in 46% herds, to lactating cows in 18% herds (of which eprinomectin was given to 64%), heifers in 82% herds and calves in 86% herds (Bloemhoff et al., 2014).

1.2.10.2.3 Non – chemical control mechanisms

Various attempts have been made to try to interfere with different stages of the lungworm lifecycle without the use of chemical products. The predacious, net-forming fungus *Duddingtonia flagrans* was able to significantly reduce the number of infective *D.viviparus* larvae on herbage surrounding faecal pats (Fernández et al., 1999). However, further work is needed to look at spore delivery (for example in medicated feed), establishing a regulatory framework and assessing the environmental impact before this is used in a commercial setting. Hypothetically, preventing the growth of *Pilobolus* spp. fungus on faecal pats would dramatically reduce transmission of infective larvae onto pasture. However, this has gathered minimal research interest to date.

Breeding for genetic resistance to parasites has proven successfully amongst small ruminants. A simulation model of *Trichostrongylus colubriformis* infection in sheep, found that resistant herds would have greatly reduced seasonal peaks in parasite burden and reduced larval numbers on pasture (Barger, 1989). Furthermore, breeding for resilience in sheep, where the same parasite burden leads to less host pathology, would lead to lambs with higher growth rates and lower dag scores compared to untreated lambs grazing infective pasture (Bisset and Morris, 1996). Less attention has been given to breeding resistance in cattle than in small ruminants, possibly because of concerns of selecting for reduced production parameters in an already high-performing cattle herd. However, numerous calf studies looking at aspects of *D.viviparus* epidemiology found that whilst grazing the same pasture, there were large inter-host variations in parasite burden (see for example Marius et al., 1979; Jørgensen, 1980b; Oakley, 1982) suggesting that host factors influence *D.viviparus* establishment. Whether this could be manipulated for parasite control is currently unknown.

1.2.11 Recent changes in lungworm epidemiology

1.2.11.1 Prevalence of disease

The prevalence of *D.viviparus* infections amongst European dairy herds has ranged from 64.4% in Ireland (Bloemhoff et al., 2015), to 21.1% in Germany (Klewer et al., 2012) and 2.9% in Switzerland (Frey et al., 2018). Within herd prevalence levels range from 8.2% to 46.8% of lactating cattle (mean 20.4%) (Schunn et al., 2012). A recent study, and one of the few to look at parasitic diseases amongst beef suckler herds, found *D.viviparus* faecal patency ranged from 0% to 17.7% of animals in the herd (Gillandt et al., 2018). Accurate prevalence levels are difficult to judge given insensitive diagnostic tests and temporal variations in seropositivity.

Current prevalence estimates in Great Britain are unknown. However, there was a significant increase in cases reported to the Veterinary Investigation Diagnosis Analysis (VIDA) during the 1990s with 1993 seeing a record number of cases (David, 1999). Recent reports suggest that this trend has continued, with a significant increase in cases reported in 2014 according to the Great Britain Cattle Disease Surveillance emerging threats quarterly report (Department for Environment Food and Rural Affairs et al., 2014). The authors report that:

“There were several cases of respiratory disease in adult cattle at grass...with lungworm not always considered as a differential diagnosis, which led to significant morbidity and in some cases mortality. This suggests that awareness of lungworm as a major cause of respiratory disease in grazing cattle may not be as high as perhaps it should be.” (Department for Environment Food and Rural Affairs et al., 2014).

In addition, the disease appears to have also extended its geographical range away from the southwest of England where the cattle density is highest, and into the north of England and Scotland. There were twice as many cases of lungworm diagnosed across Scotland in 2010 (n=12) than in 2009 (n=6) (Veterinary Services, 2010). This is further supported by anecdotal reports from the farming community with both veterinarians and dairy farmers reporting difficulties in controlling the disease and an unprecedented number of outbreaks [personal communication].

It is possible that climate change may be having some effect on the increased incidence of the disease. From 1997 – 2001, there was a significant increase in cases of ovine parasitic gastroenteritis which coincided with tangible and significant increases in temperature (range +0.8 to +1.4) in February to May and 19mm increased rainfall in April from 1998 onwards (van Dijk et al., 2008). The role that climate has had on the increased prevalence of lungworm disease has yet to be shown.

1.2.11.2 Increased number of cases in adult cattle

Traditionally, lungworm was seen as a disease of first-season grazers at grass (David, 1997). Abattoir studies from the late 1960s looked at the incidence of lungworm in cattle and found that 4.3% of cattle under 1 year-old and 1.3% of cattle over 1 year had patent lungworm infections (Gupta and Gibbs, 1969). However, case studies started to emerge from the 1980s of adult dairy cows developing clinical disease (Bateman et al., 1986) and by 1993, parasitic bronchitis was the commonest respiratory disease of adult cattle reported in Great Britain (David, 1999).

Several factors have been hypothesised as contributing towards this change in age distribution of cases. During the 1980s and 1990s, vaccine uptake decreased (David, 1997) whereas the use of anthelmintics in second-season animals tripled (Ploeger et al., 2000). An Irish study found that in 2009, 46% of dry cows were treated with anthelmintics. This figure had increased to 82% by 2011 (Bloemhoff et al., 2014). It has been hypothesised that the overuse of anthelmintics limits the opportunity of cattle to develop immunity against lungworm leading to a more vulnerable adult herd (Downey et al., 1993; Eysker et al., 1993; Ploeger et al., 1990b). Currently, any reasons behind this “epidemiological shift” (David, 1999) are purely speculative and warrant further investigation if we are to understand the likely future changes in *D.viviparus* epidemiology.

1.3 Background to the thesis

1.3.1 Thesis outline

This thesis aims to address some of the most urgent gaps in the knowledge of the epidemiology of *D.viviparus* amongst dairy cattle. Chapter 2 begins by providing a spatial and temporal assessment of cases of lungworm disease in Great Britain reported over a 40-year period. This will be the first major analysis of the scale of any “epidemiological shift” in disease prevalence, geographical distribution or age of cases reported since the 1970s. Possible causes for these changes will be hypothesised. This will act as a useful baseline study by which to assess future changes against. Chapter 3 will investigate infection dynamics of lungworm within a dairy herd to design a new, quick and cheap diagnostic test that can be used routinely to monitor herds for the circulation of *D.viviparus*. Chapter 4 will use this test on a nationwide survey of dairy farmers in Great Britain in order to understand the relevance of farm – management risk factors in the establishment of the parasite on farm. Chapter 5 will aim to understand the role that climate plays in altering local and national disease risk. A previous gastrointestinal mathematical model will be adapted for the free-living stages of the *D.viviparus* lifecycle in order to understand how the infection pressure develops on pasture influencing both the timing and severity of disease. Although a mathematical model has previously been developed for the parasitic stages of lungworm infections (Ploeger and Eysker, 2000), no models currently exist for the establishment of infective larvae on pasture. This chapter will investigate to what degree the changes observed in Chapter 2 are a response to climate changes which have already occurred, and furthermore, what changes are likely to happen under future climate change scenarios.

1.3.2 Aims and objectives for thesis

This thesis is designed to answer some of the major concerns that threaten to limit the sustainable control of *D.viviparus* under global change. In particular, the relative contribution of farm management practices compared to climate change in creating the epidemiological change will be assessed. It is hoped that this will lead towards predictions in how the risk of disease may change in the future and therefore, could lead to more informed decisions regarding sustainable *D.viviparus* control. Specific aims for the thesis are:

- 1) to quantify any changes in the “epidemiological shift” in lungworm disease across Great Britain over the past 40 years with particular reference to the spatiotemporal distribution of cases and demographic features of lungworm cases
- 2) to understand the relative contribution of farm - based practices compared to climate (change) in causing the observed changes in disease epidemiology
- 3) to understand the influence of temperature and rainfall on the development, survival and migration rates of free – living *D.viviparus* larvae
- 4) to understand likely challenges facing sustainable lungworm control under climate change
- 5) to understand which farm – based management factors increase the risk of *D.viviparus* being present on a dairy farm
- 6) to design a cheap presence – absence test for the presence of *D.viviparus* within dairy herds which can be used for the routine testing and monitoring of herds
- 7) to enhance interest and awareness of *D.viviparus* amongst researchers, veterinarians and farmers

Chapter 2: A spatial and temporal assessment of cases of lungworm disease in Great Britain from 1975 – 2014.

2.1 Introduction

The four pillars of global food security encompass the availability of food, access to food and food utilization, plus stability of these three dimensions over time (FAO, 2008). Helminth infections in ruminants are a major economic constraint for the livestock industry (Charlier et al., 2014b) and thus threaten global food security. An increased bulk tank *Ostertagia ostertagi*, *Fasciola hepatica* and *Dictyocaulus viviparus* antibody titre from the 25th to 75th percentile was associated with milk production losses of 1.1 kg per cow per day (Charlier et al., 2005a), 0.7 kg per cow per day (Charlier et al., 2007) and 0.5 kg per cow per day (Charlier et al., 2016a) respectively. Clinical lungworm disease is associated with an even higher economic burden, estimated at £100 - £116 per adult cow in the herd and milk production losses of 4 kg per cow per day (Holzhauer et al., 2011). On farms where lungworm problems have been encountered annually, with a more or less predictable seasonality, farmers will have learned to respond rapidly to clinical milk drop. However, if the epidemiology of *D.viviparus* is changing in space and / or time, observed disease may be devastating. For example, if the parasite were to emerge in a region where disease is not commonly observed then it may take a long time for the disease to be diagnosed and affected cattle treated. Anecdotally, this is currently happening in Scotland (see Chapter 1) but patterns have not been quantified. Any changes in lungworm epidemiology that make the disease more unpredictable may pose a threat to the United Kingdom's food security.

The Veterinary Investigation Diagnosis Analysis (VIDA) surveillance database collates reports from regional veterinary surveillance laboratories to monitor the incidence rates of exotic and endemic diseases of cattle, sheep, pigs and poultry in Great Britain. This is a passive surveillance system allowing voluntary submission of cases for post – mortem by farmers and veterinarians. Lungworm disease is not notifiable and so reports are subjected to reporting bias. Specifically, factors which influence a farmer’s or veterinarian’s motivation to submit samples, an altered disease awareness and changes in laboratory methods will all confound the results. Encouragingly, an analysis of the spatiotemporal changes in parasitic gastroenteritis in sheep using the same database found there were no correlations between the number of submissions per year and the market price of lamb. This suggests that motivations for submitting cases may encompass more than farm economics. Moreover, the advent of more sophisticated diagnostic techniques such as the enzyme-linked immunosorbent assay (ELISA) for use in serum (Cornelissen et al., 1997; von Holtum et al., 2008) and milk (Fiedor et al., 2009) means that cases may be more likely to be diagnosed on farm without the involvement of central laboratories. Nonetheless, cases which are clinically unusual, severe or unexpected may still be submitted, allowing the VIDA database to become a useful resource for detecting long – term temporal and spatial trends. Moreover, as farmer motivation to submit samples will be highest when the disease is emerging, it may be expected to be relatively sensitive to picking up early changes in disease epidemiology.

Despite intermittent accounts that the incidence of lungworm may be increasing (David, 1999; Department for Environment Food and Rural Affairs et al., 2014; Veterinary Services, 2010), there have been no in-depth analytical reports comparing the spatial or temporal trends of lungworm cases across the United Kingdom. Van Dijk (2004), presented a very crude

analysis of VIDA data collected up to 2004 and showed that the overall prevalence of lungworm disease had risen sharply towards the end of the Nineties. It was also shown that the increase in the number of cases was due to a rise in clinical cases in older cattle, whereas up to the early nineties it had been regarded as a disease of young stock (Graham, 1997; van Dijk, 2004). Van Dijk (2004) further reported that the Southwest of England was disproportionately represented in the number of cases (33% of all lungworm cases occurring in a region containing 22% of UK cattle) whereas Scotland was disproportionately underrepresented (8% of all cases in a region containing 24% of UK cattle). No early evidence was found for a shift in temporal patterns (seasonality of outbreaks) at the time. The crude data presented serve as a baseline. However, since this analysis, it is unknown whether the overall increasing number of cases has continued, whether the trend of more disease in adult cattle has continued and whether the spatial patterns have changed at all. The aim of this chapter is to quantify and describe recent trends in the epidemiology of lungworm in Great Britain. The seasonality, age of cattle affected and geographical distribution of cases will be compared between regions in order to quantify any changes in epidemiology. It is hoped that these trends will provide clues for future changes in lungworm disease, for example under climate change scenarios.

2.2 Methods

The VIDA database records every submission made to the regional laboratories of the Animal and Plant Health Agencies (APHA) Veterinary Investigation centres in England and Wales and the Scottish Agricultural College (SAC) in Scotland. Data was collected on the number of cases of dictyocaulosis in the database from 1975 to 2014. In order to compare the dictyocaulosis incidence trends to those of common gastrointestinal parasites over the same timeframe, cases of ostertagiosis and unspecified parasitic gastroenteritis (PGE) were also collected. The diagnostic criteria became centrally defined in 1999 but continued, as for pre-1999, to be based on the judgement of experts in veterinary pathology. Cases were classified according to Table 2.1. Seropositivity and eosinophilia alone were insufficient evidence to classify a disease. Ostertagiosis is primarily diagnosed from post-mortem samples in late winter or early spring and typically refers to the syndrome known as type 2 ostertagiosis (disease caused by the synchronous re-emergence of previously hypobiotic *Ostertagia ostertagi* larvae). Unspecified PGE in cattle is usually caused by *Ostertagia ostertagi* (type 1 disease) or *Cooperia* spp. (primarily *Cooperia oncophora*) in young calves on pasture and diagnosed ante-mortem, for example, through faecal egg testing.

Table 2.1 Case definitions of dictyocaulosis, ostertagiosis and unspecified parasitic gastroenteritis in cattle according to the Veterinary Investigation Diagnosis Analysis (VIDA) database

Parasitic disease	Case definition
Dictyocaulosis	Relevant clinical history and / or gross pathology and either: a) demonstration of <i>Dictyocaulus viviparus</i> in the bronchial tree, b) detection of first stage larvae (L1) in the faeces, or c) histopathology
Ostertagiosis	Relevant clinical history and significant numbers (approximately 20,000 or more) of <i>Ostertagia ostertagi</i> adults/larvae in the abomasum
Unspecified PGE	Relevant clinical history and / or gross pathology and / or histopathology and either: a) detection of worms in gastrointestinal tract, b) faecal worm egg count (usually >500 epg in individual FEC examinations), or c) larval culture / identification

2.2.1 Data available

The number of lungworm cases diagnosed on each date (month and year) and age of cattle was compared to the total number of cattle samples submitted to the network of laboratories (for any diagnostic reason) in each month and age range to calculate a diagnostic rate (lungworm cases per 1,000 submissions). Data on the total number of submissions was only available from 1979 onwards and so diagnostic rates were calculated from 1979. Likewise, the age categories of cases were also available from 1979. Regional data was only available from 1999 and so spatial analyses were performed using data from 1999 to 2014.

2.2.2 Overall trends in diagnoses

All statistical analyses were performed using the R statistical software (version 3.3.3). Spearman's rank correlation was used to test for significance in changes of the diagnostic rate from 1979 to 2014. To assess more detailed diagnostic rate changes over time, years were divided into seven lots of 5-year blocks (1980 – 1984, 1985 – 1989, 1990 – 1994, 1995 – 1999, 2000 – 2004, 2005 – 2009, 2010 – 2014). The Kruskal Wallis test was used to assess whether there were any significant differences between the time blocks with the Mann-Whitney U test used as a post-hoc test to assess significance between pairs. Overall trends were assessed for all three parasitic diseases: dictyocaulosis, ostertagiosis and unspecified PGE.

2.2.3 Spatial distribution of cases

From 1999 to 2014, spatial data was provided at the regional level (Wales, Scotland, southwest - , southeast - , east - , midwest - and north England) as specified in Appendix 1. To assess the relative impact of lungworm disease in each region over time and to allow for annual variations in lungworm intensity, the proportion of lungworm cases recorded in each region from the total number of lungworm cases recorded for Great Britain in that year was calculated. Changes in the proportions from 1999 to 2014 for each region was assessed using Spearman's rank correlation test.

2.2.4 Age distribution of cases

The proportion of cases diagnosed in young stock (either first season grazing calves or non-lactating heifers) out of the total number of submissions in young stock was calculated over each decade (1980 – 1989, 1990 - 1999 and 2000 – 2009) and compared with the proportion

in adult cattle (over two years old) using the chi-squared test. The odds ratio and Wald's confidence intervals of a case being under two years old was calculated for each decade.

2.2.5 Seasonal trends

The seasonal distribution of cases was assessed by calculating the proportion of the total number of cases recorded over the year that occurred in each month. Variations in seasonality from 1975 to 2014 across Great Britain and in each region were assessed using the Spearman's rank correlation test.

2.3 Results

A total of 7,616 lungworm cases were diagnosed from 1975 - 2014. There was an overall significant increase in the dictyocaulosis diagnostic rate between 1980 (0.97 cases per 1,000 submissions) and 2014 (4.04 cases per 1,000 submissions, $r_s=0.65$, $p<0.001$) (Figure 2-1). There were significant differences between the 5-year timeframes ($H(6)=27.3$, $p<0.001$). Each 5-year block between 1980 and 1994 (median 1.22 cases per 1,000 submissions) had a significantly lower diagnostic rate than blocks between 1995 and 2009 (median 7.47 cases per 1,000 submissions, $U\leq 2$, $p\leq 0.03$). There was a significant decrease in cases between 2000 - 2004 (median 9.35 cases per 1,000 submissions) and either 2005 - 2009 (median 6.18 cases per 1,000 submissions, $U=23$, $p=0.03$) or 2010 - 2014 (median 3.54 cases per 1,000 submissions, $U=25$, $p=0.01$). Most recently, a small increase in cases is noted between 2012 and 2014.

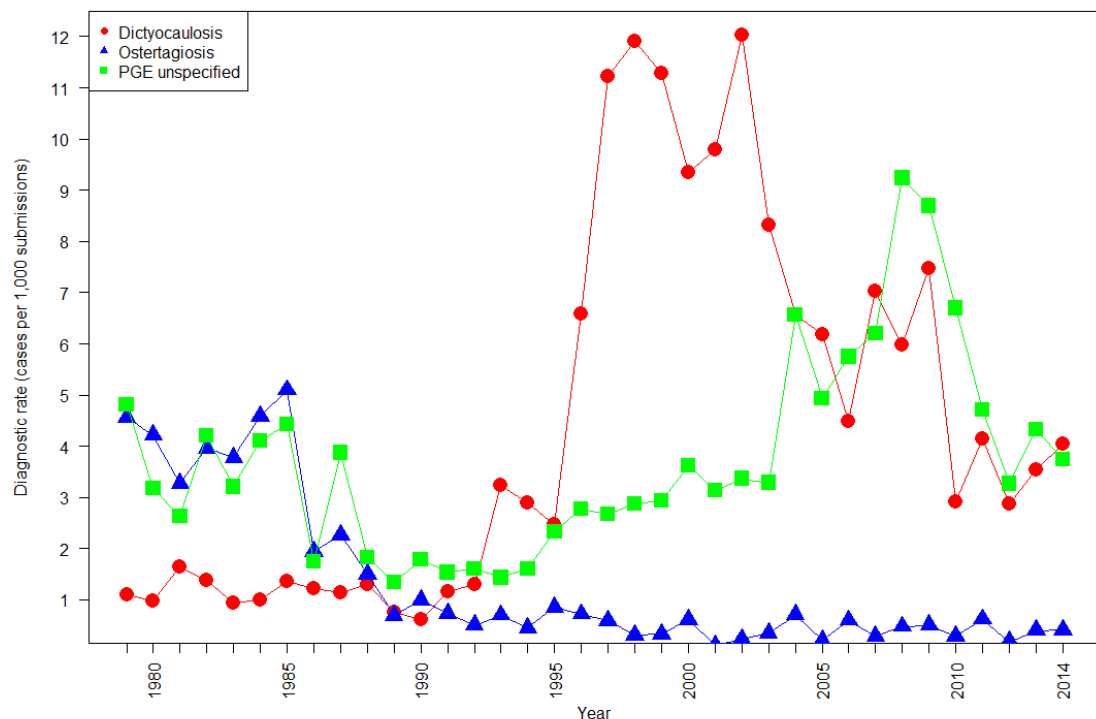


Figure 2-1 Diagnostic rate (cases per 1,000 cattle submissions) of Dictyocaulosis (red circles), Ostertagiosis (blue triangles) and parasitic gastroenteritis (green squares) from 1979 - 2014.

The diagnostic rate of ostertagiosis significantly decreased between 1980 (4.21 cases per 1,000 submissions) and 2014 (0.41 cases per 1,000 submissions, $r_s = -0.81$, $p < 0.001$). There were significant differences between the 5-year blocks in ostertagiosis diagnostic rates ($H(6) = 22.9$, $p < 0.001$) with each 5-year block between 1980 and 1989 (median 3.53 cases per 1,000 submissions) having a significantly higher rate than each 5-year block between 1995 and 2014 (median 0.41 cases per 1,000 submissions, $U \leq 23$, $p \leq 0.02$).

Rates of parasitic gastroenteritis significantly increased from 1980 (3.18 cases per 1,000 submissions) to 2014 (3.74 cases per 1,000 submissions, $r_s = 0.46$, $p < 0.001$) with significant differences between each 5-year block ($H(6) = 24.3$, $p < 0.001$). Of particular note, 5-year blocks between 1990 and 1999 (median 2.06 cases per 1,000 submissions) all had significantly lower diagnostic rates than between 2000 and 2014 (median 4.71 cases per 1,000 submissions, $U = 0$, $p = 0.01$).

Table 2.2 Correlation between percentage lungworm cases per region, and year from 1999 – 2014

Region		r_s	p value
England	- southwest	-0.82	<0.001***
	- southeast	-0.46	0.08
	- east	-0.62	0.01**
	- midwest	0.02	0.94
	- north	0.08	0.78
Wales		-0.40	0.13
Scotland		0.86	<0.001***

Significance: * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

The proportion of cases recorded from southwest and east England significantly decreased from 1999 to 2014 ($r_s=-0.82$, $p<0.001$ and $r_s=-0.62$, $p=0.01$ respectively) (Table 2.2). The proportion of cases diagnosed in Scotland significantly and strongly increased from 1999 to 2014 ($r_s=0.86$, $p<0.001$) (Figure 2-2) becoming the region with the highest proportion of cases across the country by 2009 (Figure 2-3).

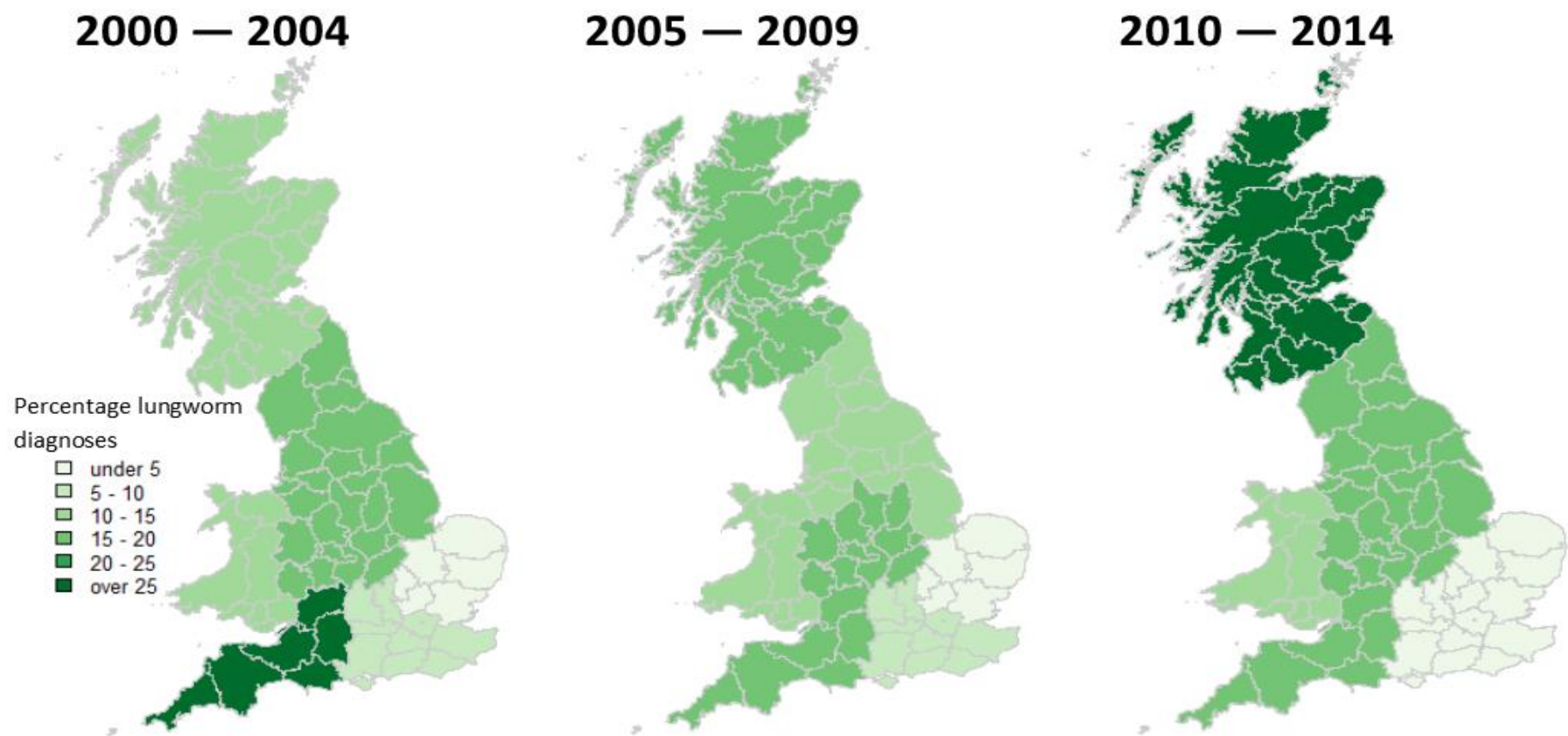


Figure 2-2 Lungworm cases recorded by the Veterinary Investigation Diagnosis Analysis database from 1999 to 2014 as a percentage of the number of cases reported each year in all regions of Great Britain



Figure 2-3 Lungworm cases recorded by the Veterinary Investigation Diagnosis Analysis database from 1999 to 2014 as a percentage of the number of cases reported each year in North England (red circles), Southwest England (blue squares) and Scotland (green triangles).

Cases of lungworm disease showed strong seasonality with (78.3%) cases diagnosed between July and October (Figure 2-4). Across Great Britain, there was a significant decrease in the proportion of cases diagnosed in January from 1975 to 2014 ($r_s=-0.36$, $p=0.02$). This was characterised by a decrease in the proportion of cases diagnosed in January from Scotland ($r_s=-0.37$, $p=0.02$) but an increase in cases in southwest England ($r_s=0.37$, $p=0.02$) and Wales ($r_s=0.35$, $p=0.03$). Southwest England also saw an increased proportion of cases diagnosed in February ($r_s=0.43$, $p=0.01$) and December ($r_s=0.32$, $p=0.05$). In Wales, there was also an increase in the proportion of cases diagnosed in November ($r_s=0.39$, $p=0.01$) and December ($r_s=0.36$, $p=0.02$). An increasing proportion of cases were diagnosed from northern England in March ($r_s=0.34$, $p=0.04$) and October ($r_s=0.55$, $p<0.001$) from 1975 to 2014. The proportion of cases diagnosed in June significantly decreased in Scotland ($r_s=-0.43$, $p=0.01$) and increased in midwest England ($r_s=0.31$, $p=0.05$) and Wales ($r_s=0.31$, $p=0.05$). There were no changes in east or southeastern England.

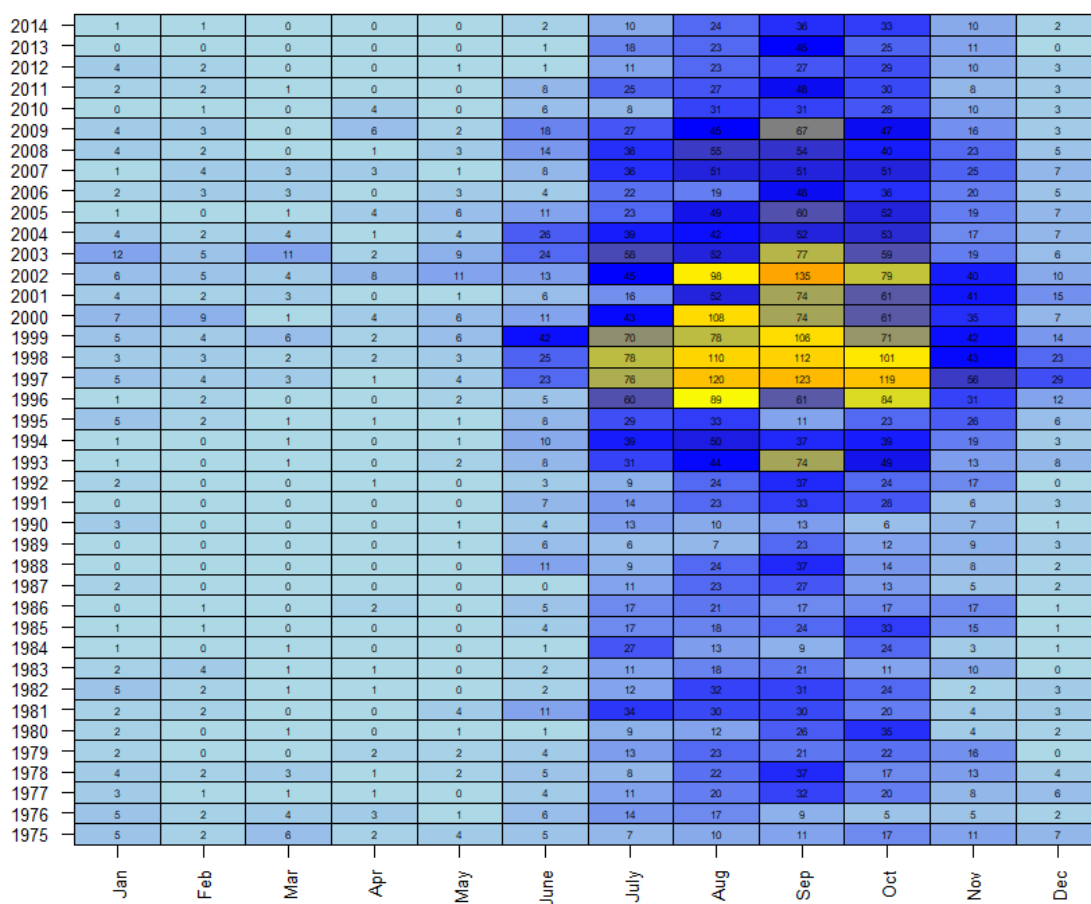


Figure 2-4 Monthly distribution of lungworm cases recorded on the Veterinary Investigation Diagnosis Analysis (VIDA) database from any region of Great Britain 1975 to 2014 (rows). Heatmap colours refer to number of cases from light blue (fewest cases) to dark orange (most cases). Numbers within cells refer to the number of cases diagnosed per month and year.

During the 1980s, the odds of a lungworm case being under two years of age was 5.22 times higher than for adult cattle (95% confidence intervals 4.20 – 6.48, $\chi^2=280.3$, $p<0.001$) (Figure 2-5). During the 1990s and 2000s, the odds of a case being under two years (3.28, 95% confidence intervals 2.96 – 3.64, $\chi^2=560.8$, $p<0.001$ and 3.37, 95% confidence intervals 3.05 – 3.71, $\chi^2=657.8$, $p<0.001$ respectively) was significantly higher than a case in adult cattle. However, these odds were lower than during the 1980s.

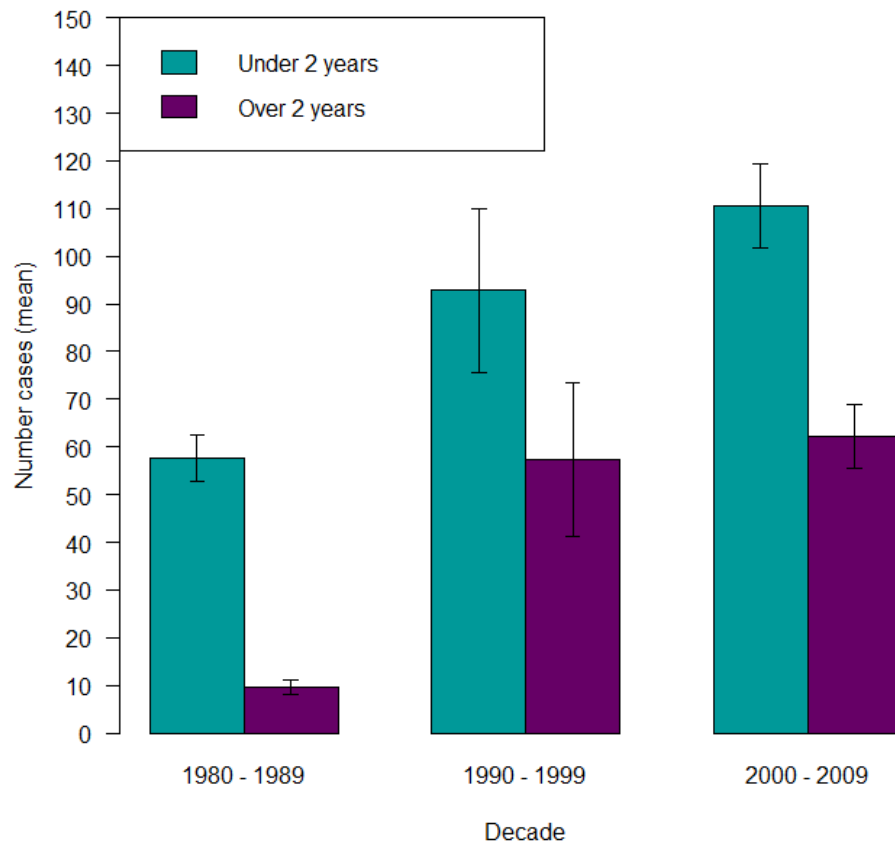


Figure 2-5 Average age of lungworm cases (mean and standard error of mean, vertical bars) recorded in the Veterinary Investigation Diagnosis Analysis (VIDA) database by decade.

2.4 Discussion

According to the Veterinary Investigation Diagnosis Analysis (VIDA) database, there was an overall increase in the diagnostic rate of lungworm across Great Britain from 1980 to 2014 with a particularly large increase from 1995 to 2000. Median diagnostic rates significantly increased from 1980 – 1994 and 1995 – 2009. There was a significant decrease in cases from 2000 – 2004 and 2005 – 2014. This may reflect a complacency in lungworm diagnosis and therefore reflects an artefact due to the passive surveillance system.

The results presented here, together with comparisons to trends in other livestock parasitic diseases, may suggest hypotheses for the changes observed. Rates of parasitic gastroenteritis also increased from 1990 – 1999 to 2000 – 2014 such that diagnostic rates between unspecified parasitic gastroenteritis and dictyocaulosis occurred at a similar rate between 2005 and 2014. According to the VIDA database, there was a similar increase in recorded cases of nematodiosis, unspecified parasitic gastroenteritis and haemonchosis cases in sheep in Great Britain during the 1990s (van Dijk et al., 2008). This coincided with a significant increase in mean monthly temperature during February to May from 1998 onwards and increased rainfall in April from 1999 (van Dijk et al., 2008). The diagnostic rate of acute and chronic disease caused by the trematode *Fasciola hepatica* has, in cattle and sheep, increased at rates very similar to those reported here for lungworm in cattle (van Dijk et al., 2010).

The proportion of cases diagnosed in southwest England and eastern England has decreased since 1999 whereas the proportion of cases in Scotland has increased dramatically seeing the highest proportion of cases across the country by 2009. The increase in cases in northern England and Scotland from 2009 onwards fits with reports from the Scottish Agricultural

College that there were twice as many cases of lungworm diagnosed in 2010 than in 2009 (Veterinary Services, 2010). Disease caused by the three major categories of parasitic gastroenteritis in sheep (nematodiosis, unspecified parasitic gastroenteritis and haemonchosis) were also shown to increase in Scotland from 1975 to 2006 (van Dijk et al., 2008). The more northerly spread of parasitic diseases in ruminants is an interesting phenomenon and could suggest a role for climate change. Lungworm disease outside of Great Britain shows similar trends. Significant geographical clusters were found to have a higher incidence of dictyocaulosis in Swiss dairy herds (Frey et al., 2018), suggesting that geographical clustering of risk factors can be responsible for disease incidence. However, a German study investigating the role of climate and type of pasture on antibody titres within bulk tank milk samples failed to find any significance for meteorological variables, finding only the presence of lentic water bodies and proportion of grassed area significant (Frey et al., 2018). However, this study found the addition of a random intercept for spatial information enhanced the overall model fit, suggesting a role for a perhaps unmeasured, geographically clustered risk factor(s). It is likely therefore, that significant spatial risk factors, such as meteorological conditions, have impacted the incidence of several parasitic diseases in ruminants across Europe. More work is needed to specifically understand the role that this has had in altering the spatial and temporal incidence of lungworm disease across Great Britain and to quantify the effects of temperature and precipitation changes on each life cycle stage of *D.viviparus*.

The apparent change in seasonality of lungworm disease could also be due to the effects of climate change. There is evidence that more cases are being diagnosed in the winter, particularly December to February in southwest England, November to January in Wales and March in northern England. The trends also suggest that the highest risk periods for

lungworm (typically coinciding with the Autumn months), may be expanding into June in midwest England and Wales and October in northern England. This could imply that the disease is becoming more unpredictable, with the potential for sporadic disease outbreaks if the climate is conducive to optimal transmission on pasture. Exceptions to this are in Scotland where disease is becoming less frequent in January and June.

Although some changes can be explained by the effects of climate change, this fails to explain all of the epidemiological changes seen in lungworm disease since 1975. Diagnostic rates of type 2 ostertagiosis declined during 1980 to 1999, suggesting either that climate conditions had become unfavourable to disease transmission; that farmers were less likely to report cases of the disease; or that farm management practices had changed sufficiently to decrease the risk of ostertagiosis cases. The key reason for this may be the introduction of avermectins, which were heavily advocated for use at housing since the Nineties. A survey performed by the Veterinary Laboratories Agency from May to December 1996 found that 27 out of 32 herds who experienced lungworm outbreaks used anthelmintics in second season calves with five herds using anthelmintics in the first three seasons (David, 1997). The author further argues that this represents an increased anthelmintic usage from the previous 10 to 15 years. This effect was not exclusive to the UK. Anthelmintic usage in second grazing season animals in the Netherlands tripled from the mid-1980s to 1997 (Ploeger et al., 2000). The present study found that cattle under two years old are still the most likely to be diagnosed with lungworm, but the odds of a case in young cattle have decreased since the 1980s. The reduced immunity in older cattle from the 1990s onwards may be explained by reduced opportunities to develop a natural immunity when cattle are overprotected with anthelmintics (Borgsteede et al., 1998; David, 1997; Ploeger et al., 2000). Adding to this situation is the possibility of anthelmintic resistance. Although this is a major problem in

sheep, anthelmintic resistance has not been reported for *D.viviparus* in cattle. However, although calves treated with ivermectin, doramectin or fenbendazole during one grazing season displayed mild clinical signs of disease with some faecal patency during challenge studies in the following grazing season, they did show considerable resistance to the disease compared to untreated controls (Downey et al., 1993; Schnieder et al., 1996a; Taylor et al., 2000). Although it is tempting to blame the increase in cases amongst adult cattle on the excessive use of anthelmintics, this may be over – simplifying the situation.

Use of the lungworm vaccination is a major proven method of improving herd immunity. Sales of the vaccine have declined in the United Kingdom since the 1980s (van Dijk, 2004) with a survey of the UK farming industry in 1996 finding that only three out of 32 herds with clinical lungworm outbreaks used the vaccine and two of the three herds only saw clinical disease in unvaccinated animals (David, 1997). During the 1990s, only 20 – 25% of dairy herds used the vaccine whereas in Scotland, where the disease incidence was lower, this figure was closer to 50% (David, 1999). After the bovine spongiform encephalitis (BSE) epidemic in the UK, the lungworm vaccine was withdrawn from the Netherlands in 1996 which led farmers to adopt other preventative measures such as long acting anthelmintic treatments (Borgsteede et al., 1998). This withdrawal of the vaccine coincides with the timing of the major peak in lungworm incidence from 1996 to 2003. In order to understand the impact that declining vaccination use has had on the incidence of lungworm disease, further studies are needed to quantify the current uptake of the vaccine and whether there remains any spatial differences in vaccination use. In particular, qualitative studies are urgently needed to investigate motivations and barriers behind the use of the vaccination.

There are several limitations in using a passive surveillance system to make inferences on disease incidence rates. The large increase in lungworm cases from 1995 to 2003 may have increased awareness of the disease amongst farmers and veterinarians thereby encouraging further case reporting. The significant decline after 2005 may reflect a degree of reporting bias and a complacency amongst the farming community to the new epidemiological situation representing less urgency to diagnose cases. During the 1990s, research interest in lungworm disease moved away from the clinical effects in individual cattle and towards the economical side of reduced weight gain and growth performance in calves (Boon and Ploeger, 1996; Ploeger et al., 1990b, 1990c). This may have created an environment where farmers would be more willing to submit cases for diagnosis by the VIDA laboratories in order to limit some of the more widely recognised economic costs of the disease. At the same time, studies had begun to look at designing newer, more sophisticated diagnostic methods for lungworm disease once the diagnostic antigen encoded by the gene fragment Dv 3-14 was found to be a major sperm protein of *D.viviparus* (Schnieder, 1993a). Furthermore, the year 1993 saw a high number of studies developing a novel avermectin group compound and describing the excellent clinical efficacy against *D.viviparus* (Eddi et al., 1993; Goudie et al., 1993b; Jones et al., 1993; Weatherly et al., 1993) with eventual licensing as eprinomectin in 1997 (Veterinary Medicine Directorate, 2013). The increased awareness of the economics of the disease, together with increased publicity around the new treatment and new diagnostic possibilities, could have increased awareness of the disease amongst the farming community and may explain the increased submission of cases to the VIDA laboratories during the 1990s. However, these effects are likely to be only transitory and so are unlikely to explain the continued high diagnostic rates after 2003.

2.5 Conclusion

According to the Veterinary Investigation and Diagnosis Analysis (VIDA) database, there has been an increase in the diagnostic rates of lungworm disease across Great Britain from 1980 (0.97 cases per 1,000 submissions) to 2014 (4.04 cases per 1,000 submissions) with a significant increase in cases located in northern England and Scotland from 2009. There is evidence of a shift in the temporal pattern of cases which could imply that the disease is becoming more unpredictable. The reasons for the change in epidemiology of lungworm disease since the 1970s is likely to be multifactorial although similar trends and increased disease incidence amongst parasitic diseases of sheep have coincided with a significant increase in monthly temperatures from February to May. There is evidence that the 1990s represented a decade of heightened awareness of lungworm disease, as evidenced by an increased incidence rate, larger emphasis on economic consequences, enhanced diagnostic tests and new treatments becoming available. These factors may have enhanced the reporting bias within the passive surveillance system and may help to explain the rapid increase in lungworm cases from 1996 to 2003. However, these effects are likely to have only been transitory and fail to explain the continued high diagnostic rates from 1995 to 2009 compared to 1980 to 1994. Together with an increased incidence of disease, there has been a significant increase in cases amongst adult cattle from 1990 onwards. Anthelmintic use on farms has increased since the 1980s and coincided with declining uptake of the lungworm vaccine. The overuse of anthelmintics could have limited opportunities for cattle to develop a natural immunity towards lungworm, therefore increasing the naivety of the national herd and helping to explain the large increase in lungworm cases during the mid – 1990s. Further work is needed to a) understand the role of farm management practices in altering a herd's risk of acquiring lungworm disease and b) quantify the impact that small changes in temperature and rainfall would have on the life cycle stages of *D.viviparus*. Chapters 4 and 5 respectively will attempt to answer these questions.

Chapter 3: A pooled heifer milk presence-absence test for the herd diagnosis of *Dictyocaulus viviparus*

3.1 Introduction

Disease caused by the bovine lungworm, *Dictyocaulus viviparus*, is a leading cause of morbidity and mortality in grazing dairy cattle (David, 1999). Milk production losses due to lungworm disease have been estimated at 4kg/cow/day for clinical outbreaks (Holzhauer et al., 2011) with subclinical infections carrying an average estimated loss of 0.5kg/cow/day (Charlier et al., 2016a). In other European countries, using insensitive bulk tank milk tests, dairy herd prevalence levels were shown to vary between 2.9% in Switzerland (Frey et al., 2018), 9% conventional herds and 18% organic herds in Sweden (Höglund et al., 2010), 21.1% in Germany (Klewer et al., 2012) and 62.8% in Ireland (Bloemhoff et al., 2015). Robust estimates are lacking for UK grazing herds but are may be similar to the Irish estimate. Clinical outbreaks in Great Britain significantly increased from 1979 to 2014 (Chapter 2).

On any given dairy farm, lungworm may be present, with or without clinical outbreaks being witnessed, or absent, with or without a previous diagnosis of lungworm presence. While knowing the lungworm status of a farm is pertinent to the design of appropriate and sustainable parasite control strategies, many UK herds are currently unsure whether *D.viviparus* is present. A major obstacle in controlling the disease in commercial settings is the relative insensitivity of the available diagnostic tests. An ELISA based on the major sperm protein (MSP) was first developed by von Holtum *et al* (2008) and adapted for use in milk samples by Fiedor *et al* (2009). Initial studies found bulk tank milk testing to have a favourable sensitivity and specificity (100% and 97.3% respectively) when within-herd seroprevalence

levels exceeded 20% (Schunn *et al.*, 2012). However, later studies did not support these findings, instead finding lower sensitivity and specificity values of 83.3% and 95.2% which further decreased to 55.6% and 93.3% when the within-herd prevalence level decreased to 10% (Ploeger *et al.*, 2014). Low herd prevalence levels, characterised by short periods of seropositivity in individual animals, are common findings in natural infections of adult dairy cattle on pasture (Strube *et al.*, 2017). Animals which have previously been exposed do not show marked increases in antibody levels when re-exposed (Strube *et al.*, 2017). Bulk milk samples therefore may be falsely negative, especially outside of peak windows of parasite transmission.

Testing individual animals has been advocated to detect the presence or absence of *D.viviparus* within herds (Ploeger *et al.*, 2012). Serum testing of six randomly selected heifers was sufficient to be 95% confident to detect at least one positive animal within herds coughing at grass in the Netherlands (Ploeger *et al.*, 2012). At current UK laboratory rates, even testing 6 individual heifers would cost between £70 and £140 whereas any veterinary involvement in serum sampling would be higher.

In order for a presence-absence test to be widely employed in the field, it would have to be cheap, quick (preferably milk based) and (to increase test sensitivity) include samples of animals at risk only. It was hypothesised that sampling a pooled milk test from the highest antibody-producing animals in the herd would prevent the dilution effect seen in bulk tank testing, possibly caused by the large number of animals with low antibody titres. Repeated longitudinal sampling of individual animals was carried out to determine which animals in the herd have the highest antibody titre at different stages of the grazing season. Pooled milk samples were then tested and subsequently validated in a cross-sectional study of UK dairy herds.

3.2 Methods

This study was reviewed and approved by the University of Liverpool Veterinary Research Ethics committee (VREC 431).

3.2.1 Study 1: Longitudinal study design and selection of farms

A sample size calculation (number of cows to be sampled per herd) was performed on individual serum sample data derived from Swedish dairy herds. Applying the same ELISA used in this study, for two consecutive years (1999-2000) Höglund (unpublished data) sampled 39-59 individual adult cows on 5 different farms, one sampling round taking place in spring (end of April/ start of May) and another in autumn (end of October/ November), giving 20 samplings in total. For all samplings, the mean titre and 95% confidence limits (CLs) were bootstrap sampled (1000 iterations) and a significant difference in titres was defined as the 95% CLs not overlapping. For all thus identified between-farm significant differences in titres as well as for within-year significant differences on a farm (e.g. between spring and autumn) the number of animals to be included in a sampling for the confidence limits not to overlap was assessed in increments of 10 animals (e.g. 10, 20, 30 and, where possible, 40 animals). The number of farms to be sampled to be more than 95% confident to detect within-year rises in titres was calculated from the proportion of the total of 10 samplings resulting in a significant rise, using Monte Carlo analysis (1000 iterations).

Guided by the power calculation, four farms were recruited and antibody levels in individual animals assessed for the start and later parts of the grazing season. Inclusion criteria for participation consisted of the herd containing a minimum number of 100 lactating cattle, a veterinary diagnosis (through faecal, bulk tank milk sampling, serum sampling or post-mortem testing) of *D.viviparus* on the farm within the past 5 years, and lactating cattle

grazing pasture during the summer months (at least July to September). Inclusion criteria further included monthly milk recording. All herds were recording using National Milk Records (NMR) services.

A cohort of 40 first-lactation heifers (hereafter referred to as 'heifers') and 49 milking cattle parity two or above (hereafter 'cows') were randomly selected from each farm using a computerised random number generator. If fewer than 40 heifers and 49 cows were milked on any occasion, then all available heifers or cows were included. Animals who missed a month's milk recording through cessation of lactation, illness or removal from the herd were replaced by another randomly selected animal within the same age group (heifers or cows). Individual milk samples from the cohort on each farm were collected at monthly intervals during August, September and October 2016, plus once in May 2017.

3.2.2 Milk sample processing and testing

Milk samples were defatted by centrifuging at 2,000g for 15 minutes before separating the supernatant from the lipid layer. Individual milk samples had been pre-treated with the preservative bronopol, according to NMR's policy. Defatted samples were stored at -20°C until tested.

All samples were tested using a prototype of an ELISA plate developed by Boehringer-Ingelheim Svanova (Uppsala). The ELISA procedure is a solid phase indirect ELISA. Plates were coated in *D.viviparus* non-infectious major sperm protein (MSP) antigen and incubated overnight at room temperature. Positive and negative serum controls were diluted 1:100 in PBS containing 0.05% Tween-20 (PBS-Tween). Plates were incubated for 1 hour at room

temperature with 100 µl/well pre-diluted positive and negative controls and undiluted skimmed milk. After washing in a plate washer using PBS-Tween and tapping dry, plates were incubated with 100 µl/well horseradish peroxidase-conjugated anti-bovine IgG for 1 hour at room temperature. Plates were washed as before, tapped dry and incubated with 100 µl/well tetramethylbenzidine in hydrogen peroxide solution for 10 minutes in the dark at room temperature. The enzymatic colour reaction was stopped by adding 50 µl/well 2M sulphuric acid. Optical densities were then read on a microplate photometer within 10 minutes at a wavelength of 450nm. Results were expressed as an optical density ratio (ODR) using the following formula:

$$ODR = \frac{OD_{test\ specimen} - OD_{blank}}{OD_{positive\ control} - OD_{blank}} \quad \text{Equation 1}$$

For pooled-milk testing, a 1ml sample was pipetted from each individual milk sample whilst under constant homogenisation using an electronic stirrer. The pooled-milk sample underwent constant homogenisation for two minutes prior to testing according to the ELISA protocol.

3.2.3 Designing a pooled – milk test

The pooled – milk test was designed to be used on the age group of animals with the highest antibody titres. The proportion of heifers and cows who had an ODR under 0.1, between 0.1 and 0.2, and over 0.2 was compared using the chi-squared test. ODR levels between different months was compared using the Kruskal Wallis and Mann-Whitney U test.

The pooled-milk test, P_n , would be based on mixing a 1ml milk sample from n number of heifers or cows depending on which age group had the highest antibody titres in the

longitudinal study. The number of samples n could be between 1 and 20. An upper limit of 20 was set as it was considered that pooling more than 20 samples would prove too impractical for routine testing. It was also a prior assumption that P_{10} would be both practical and easy to facilitate for a commercial test.

For initial test development, the effect of creating and testing P_n where $1 \leq n \leq 20$ was simulated by bootstrap sampling, with replacement, the ODR results from n individuals in each herd and month in the longitudinal study and calculating the mean ODR. Mean individual ODR have previously been shown to correlate strongly to the bulk tank ODR (Schunn et al., 2012) and so the mean ODR from bootstrapped individual samples in our study was assumed to correlate closely with the pooled-milk test on 20 animals. The bootstrap process was repeated 1,000 times to calculate the median ODR and 95% confidence intervals. The widths of the confidence intervals in each herd, month and value of n was taken as separate data points. The Kruskal Wallis and the Mann-Whitney U post-hoc test, were used to assess the significance between different pairs of n . The minimum number of samples which did not significantly widen the confidence intervals from P_{20} was noted as P_s .

3.2.4 Study 2: Cross-sectional study: Determining the test performance under field conditions

In order to understand the performance of the pooled-milk test under field conditions, a second study, based on cross-sectional sampling of the Tesco Sustainable Dairy Group (TSDG) herds, was performed. The TSDG is a group of 600+ high yielding Holstein-breed dairy herds in England, Scotland and Wales, of which 10% operate a zero-grazing system. A questionnaire was sent to all grazing herds to gain consent for the study. This also allowed a binary

classification of the herd into either those that had or had not had a veterinary diagnosis of lungworm within the previous 2 years. In addition, herds who had displayed suggestive clinical signs of lungworm disease within the past 2 years, as reported by the farmer on the questionnaire, were noted. Individual and bulk tank milk samples from grazing herds were collected once during September to November 2017. In addition, zero-grazing herds milk recording with NMR and whose milk samples were available for scientific use, had their individual and bulk tank milk samples collected once during January to March 2018. For all grazing and zero-grazing herds, P_s and P_{10} were created and tested along with the bulk tank milk sample.

To explore the effect of varying the positivity ODR cut-off value, a receiver-operating characteristic (ROC) curve analysis, as has previously been described (Schunn et al., 2012), was used to compare P_s , P_{10} and the bulk tank milk sample. True positive herds were assumed to be grazing herds which had received a veterinary diagnosis of lungworm within the preceding 2 years whereas true negative herds were assumed to be the zero-grazing herds. The area under the curve (AUC), sensitivity, specificity and likelihood ratios of the pooled-test were calculated. The optimum cut-off was taken as that which maximised both sensitivity and specificity according to the following formula:

$$Test\ performance_{cutoff\ x} = \frac{(Sensitivity_x + Specificity_x)}{2} \quad \text{Equation 2}$$

3.3 Results

3.3.1 Study 1: Longitudinal study design and selection of farms

3.3.1.1 Sample size calculations

For both years, in spring, significant differences in mean antibody titres were identified between farms 1 and 3 (both year 1 and year 2; Table 3.1). Significant within-year differences were measured on all five farms, albeit not for both years on all farms (6 out of 10 occasions). In autumn, confidence intervals did not overlap for farms 1 and 2 (year 1) and farms 2 and 3 (year 2). Bootstrap analyses of numbers of animals to include found that the confidence intervals would overlap at the 20-animal level whereas the 30-animal level was the first to identify significant differences between populations / measurements. Thus, it was concluded that sampling at least 30 animals per herd/age group would be enough to detect differences where they exist. The minimum number of farms to be sampled to be more than 95% sure to detect within-year rises in titres was four (mean estimated number of rises detected 2.48 (95% confidence intervals 1 -4)).

Table 3.1 Bootstrapped mean *Dictyocaulus viviparus* serum antibody titres (1000 iterations) in five Swedish dairy herds, presented data includes all animals sampled. Farms were sampled on two occasions (spring and autumn) and for two years (1999 and 2000).

Dairy Farm	Year	Spring titre ^a Mean (95% CI)	Month sampled	Number animals sampled	Autumn ^b titre Mean (95% CI)	Month sampled	Number animals sampled
1	1	4.5 (3.2 – 5.8) *	April	52	5.6 (4.2-6.9)	December	54
1	2	1.8 (1.3-2.3) * †	April	59	4.4 (2.9-6.0) †	November	56
2	1	3.3 (2.3-4.3) †	May	35	9.1 (7.6-10.6)* †	November	44
2	2	6.3 (1.8-10.7)	April	44	2.8 (1.9-3.8)*	November	55
3	1	2.2 (1.7-2.6)* †	May	46	9.3 (7.4 -11.2) †	November	54
3	2	5.4 (2.6 – 8.2)*	May	45	7.6 (4.7-10.6)	October	55
4	1	2.7 (1.6 – 3.6) †	April	38	4.9 (3.7-6.1) †	October	36
4	2	1.2 (0.6 – 1.9)	April	32	6.0 (1.3-10.8)	September	43
5	1	3.4 (2.0 – 4.7) †	April	47	8.9 (6.5-11.2) †	December	48
5	2	3.7 (2.3 – 5.1) †	May	46	8.0 (6.2-9.7) †	December	55

* significance between years ($p < 0.05$)

† significance within years ($p < 0.05$)

^a Spring sampling refers to sampling in April or May

^b Autumn sampling refers to sampling in October or November

NB: Antibody titres expressed as Percentage Positive rather than OD values.

3.3.1.2 Farm demographic data

Four farms, meeting the inclusion criteria, were selected for the initial longitudinal study and more than 30 animals were sampled per age group on each occasion. The average number of lactating animals per herd was 231.3 (median 180, range 165-400); of which 69.0 were heifers in their first lactation (median 45.5, range 35-150) and 162.3 were cows of parity 2 and above (median 142.5, range 114 - 250). These herd sizes were representative of the

average size of UK dairy herds in 2016 (AHDB Dairy, 2017). A total of 1,114 individual milk samples (501 from heifers and 613 from cows) were tested from August 2016 to May 2017 (Table 3.2). It was not possible to collect herd B's milk samples in May 2017 and so their April milk samples were tested. Additionally, one herd in September, October and May did not have any milk samples available for testing.

Table 3.2 Numbers of heifers and cows per herd which were sampled in a longitudinal study to understand Dictyocaulus viviparus antibody titres in milk samples

Farm	Total number in herd (heifers / cows / total)	Number of cattle sampled per month (heifers / cows / total)			
		August 2016	Sep 2016	Oct 2016	May 2017
A	51 / 114 / 165	40 / 47 / 87	0 / 0 / 0	40 / 49 / 89	40 / 48 / 88
B	40 / 135 / 175	34 / 48 / 82	36 / 44 / 80	38 / 46 / 84	40 / 48 / 88
C	150 / 250 / 400	40 / 47 / 87	40 / 47 / 87	40 / 48 / 88	0 / 0 / 0
D	35 / 150 / 185	37 / 48 / 85	36 / 47 / 83	0 / 0 / 0	40 / 46 / 86
Mean*	69 / 162.3 / 231.3	37.8 / 47.5 / 85.3	37.3 / 46 / 83.3	39.3 / 47.7 / 87	40 / 47.3 / 87.3

**Herds where zero animals were sampled were excluded from mean calculations*

3.3.2 Age and temporal effect on antibody titres

A significantly higher percentage of cows than heifers had a milk ODR below 0.1 (82.2% and 70.4% respectively, $\chi^2(1)=21.5$, $p<0.001$) (Figure 3-1). There was no significant difference in the percentage of heifers and cows with an ODR between 0.1 and 0.2 (17.3% and 14.5% respectively, $\chi^2(1)=1.1$, $p=0.29$). A significantly higher percentage of heifers than cows had an ODR above 0.2 (12.6% heifers and 3.1% cows, $\chi^2(1)=34.9$, $p<0.001$). The average ODR amongst heifers was 0.13 (median 0.07, 25th – 75th quantile: 0.04 – 0.12) compared to 0.07

in cows (median 0.06, 25th – 75th quantile: 0.03 – 0.09). Heifers were therefore selected as the age group to enter into the pooled-milk test.

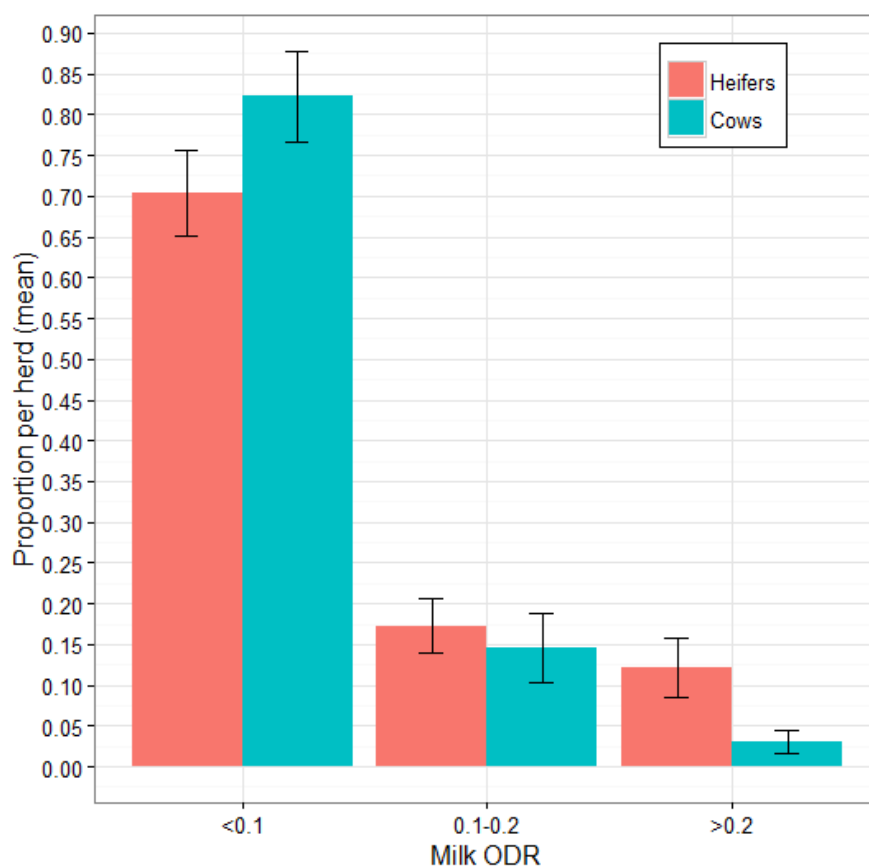


Figure 3-1 Average proportion of heifers (orange) and cows (green) per farm in each milk ODR category per herd. Vertical error bars show standard error of mean.

Milk ODR was significantly affected by the month of sampling in heifers ($H(3)=14.3$, $p=0.003$) and in cows ($H(3)=115.8$, $p<0.001$) (Figure 3-2). For cows, milk ODR were higher in May (median 0.09) than in either August (median 0.05), $U=6168$, $p<0.001$, or September (median 0.04), $U=3323$, $p<0.001$. In cows, milk ODR were lowest in September (median 0.04), which was lower than in either August (median 0.05), $U=15184$, $p=0.01$ or October (median 0.05), $U=8226$, $p=0.02$. For heifers, milk ODR were significantly higher in May (median 0.09) than in either August (median 0.07), $U=7197$, $p=0.004$ or October (median 0.06), $U=4901$, $p<0.001$. The range of ODR values was greater for heifers than for cows. In

cows, the interquartile range varied from 0.04 in August and October, to 0.05 in September and 0.06 in May. However, the interquartile range in heifer milk samples was narrow in October (0.04) but higher in May (0.06), August (0.07) and September (0.12).

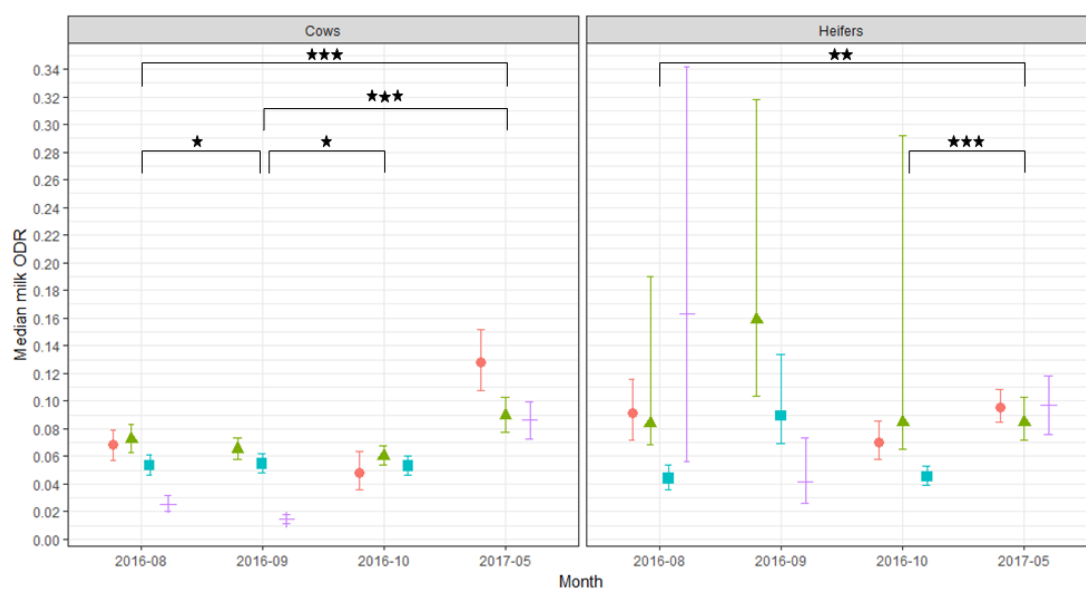


Figure 3-2 Milk ODR results from 4 UK farms sampled from August 2016 to May 2017. Points indicate median values with 95% confidence limits as vertical bars. Different colours and shapes relate to different herds. Significant values between months as detected by Mann Whitney U test where * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

3.3.3 Calculating the optimum number of milk samples to enter the pooled-milk test

The effect of increasing the number of bootstrapped heifer samples entering the pooled-milk test (P_n) on the narrowing of ODR distributions is demonstrated in Figure 3-3.

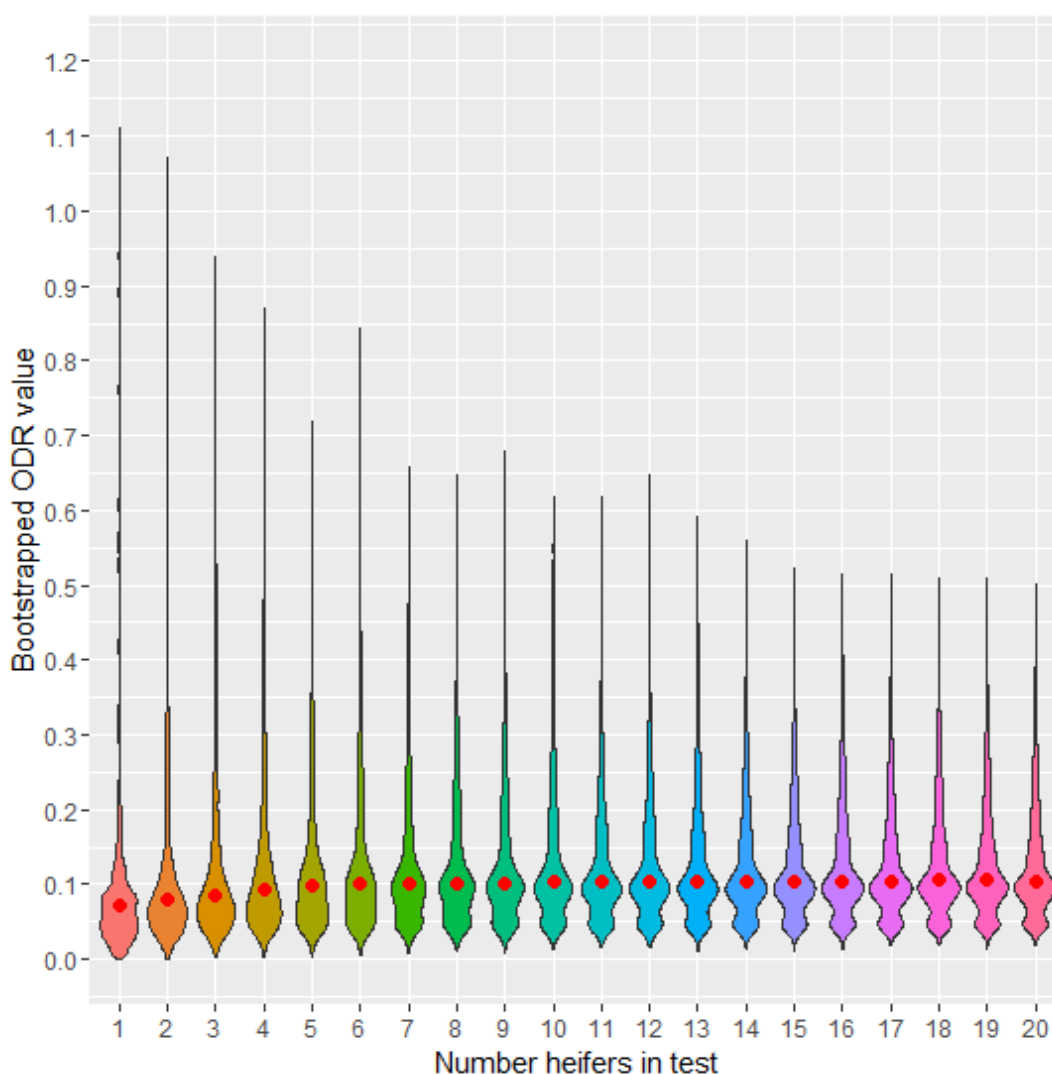


Figure 3-3 Distributions of ODR values when between 1 and 20 heifer milk samples were pooled and tested from all months (via bootstrap analysis with 1000 iterations). Red dots show median values.

Although the median bootstrapped value changed by only 0.03 when different P_n values were used, the widths of the 95% confidence intervals were significantly related to P_n

($H(19)=54.1$, $p<0.001$). Across all herds and months, the narrowest confidence interval widths occurred when P_{20} whereas $P_{n\leq 5}$ created significantly broader confidence limits than P_{20} (Figure 3-4). Thus, the minimum number of heifers to be included was six. The relative decrease in confidence intervals is small if more than 9-10 samples are included (Figure 3-4). For further validation of the test, a 6-heifer test and a 10-heifer test were compared.

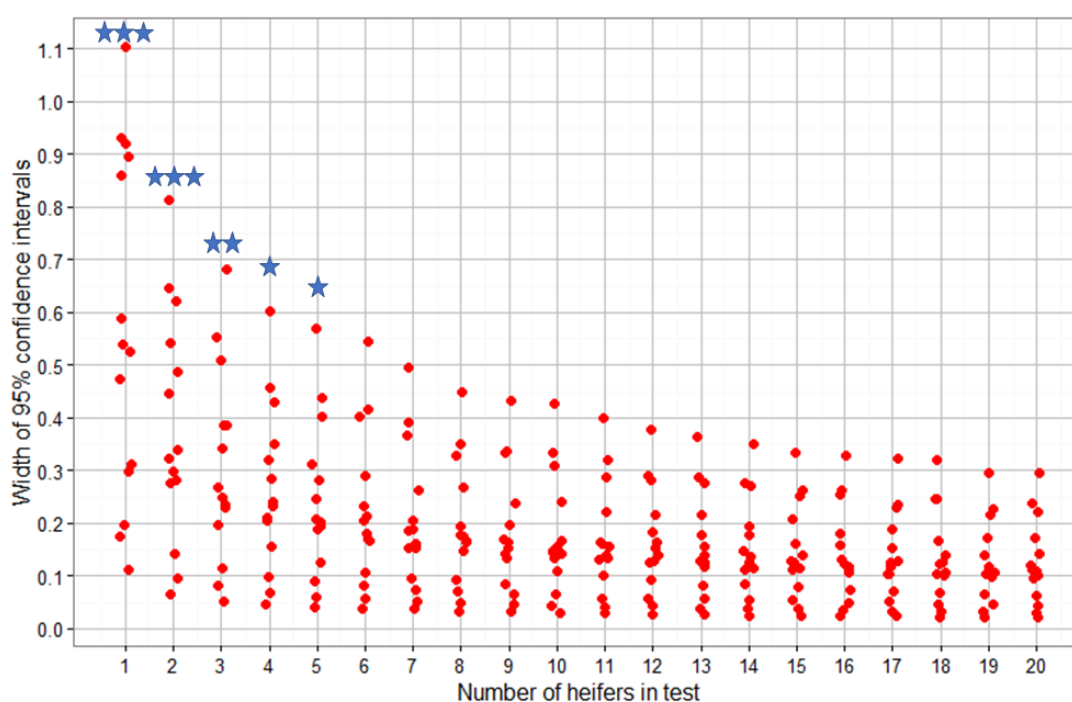


Figure 3-4 Widths of the 95% confidence intervals when between 1 and 20 heifers are randomly selected to enter the pooled-milk test. Each red dot refers to the size of the confidence interval when heifers from 1 farm and 1 month were selected and bootstrap sampled 1,000 times. Dots offset for clarity. Blue stars relate to groups of widths which are significantly wider than selecting 20 heifers according to Mann-Whitney U test (* $p\leq 0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$).

3.3.4 Study 2: Evaluating the dynamics of the pooled-milk test in a cross-sectional study

A total of 148 grazing herds from across the UK consented to inclusion in the bulk and pooled-milk testing although four herds did not have a representative bulk tank sample and 52 herds did not provide individual cow samples for pooled-milk sampling. Furthermore, two herds did not have 10 heifers and so only the 6-heifer pooled-milk sample were available. This left

a total of 90 grazing herds where bulk tank, 6-heifer and 10-heifer pooled-milk samples were available. Out of these 90 herds, 28 farmers had reported seeing suggestive clinical signs within the preceding 24 months with a further 17 farmers reporting clinical signs within the previous two to five years. Out of the 90 herds, 25 reported that they had received a veterinary diagnosis of lungworm within the preceding 24 months (Figure 3-5). Milk samples from an additional 25 zero-grazing herds were accessible for inclusion in the pooled – milk and bulk tank testing.

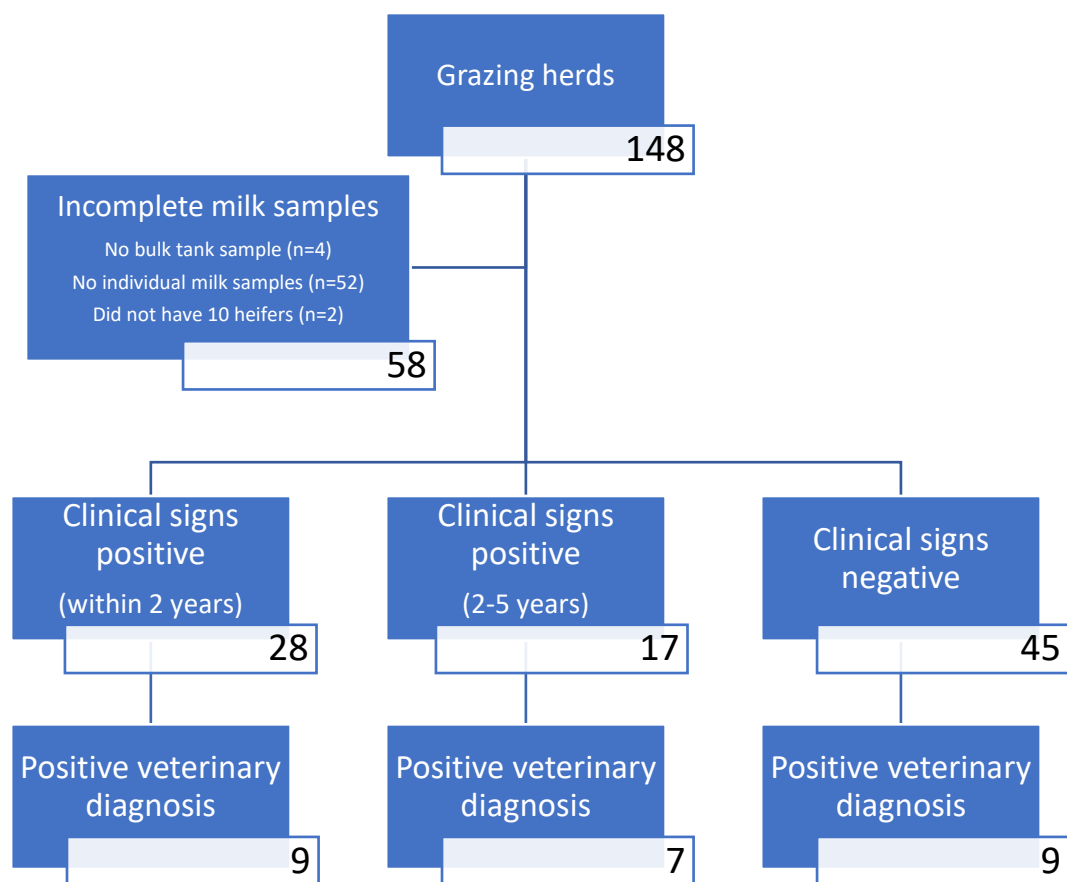


Figure 3-5 Flowchart depicting clinical status of grazing dairy herds entering cross-sectional study to decide the test performance of the pooled – heifer test under field conditions.

3.3.4.1 Determining the optimum ODR cut-off value using receiver-operating-characteristics analysis

Results from the receiver-operating-characteristics (ROC) analysis can be seen in Figure 3-6. The area under the curve (AUC) for P_{10} is high at 0.87 and shows maximal sensitivity and specificity at a cut-off of 0.16 (66.7% and 95.5% respectively) (Table 3.3 Sensitivity, specificity and likelihood ratios for the 10-heifer test (P_{10}) AUC=0.87). P_6 has a lower AUC of 0.85 and has a maximal cut-off of 0.14 (sensitivity 79.2%, specificity 72.7%) (Table 3.4). The bulk tank performs less favourably than either of the pooled-milk tests with maximal sensitivity and specificity of only 37.5% and 63.6% respectively at a cut-off of 0.18. The AUC for bulk tank testing was 0.45 and so evidently non-diagnostic in these herds (Table 3.5).

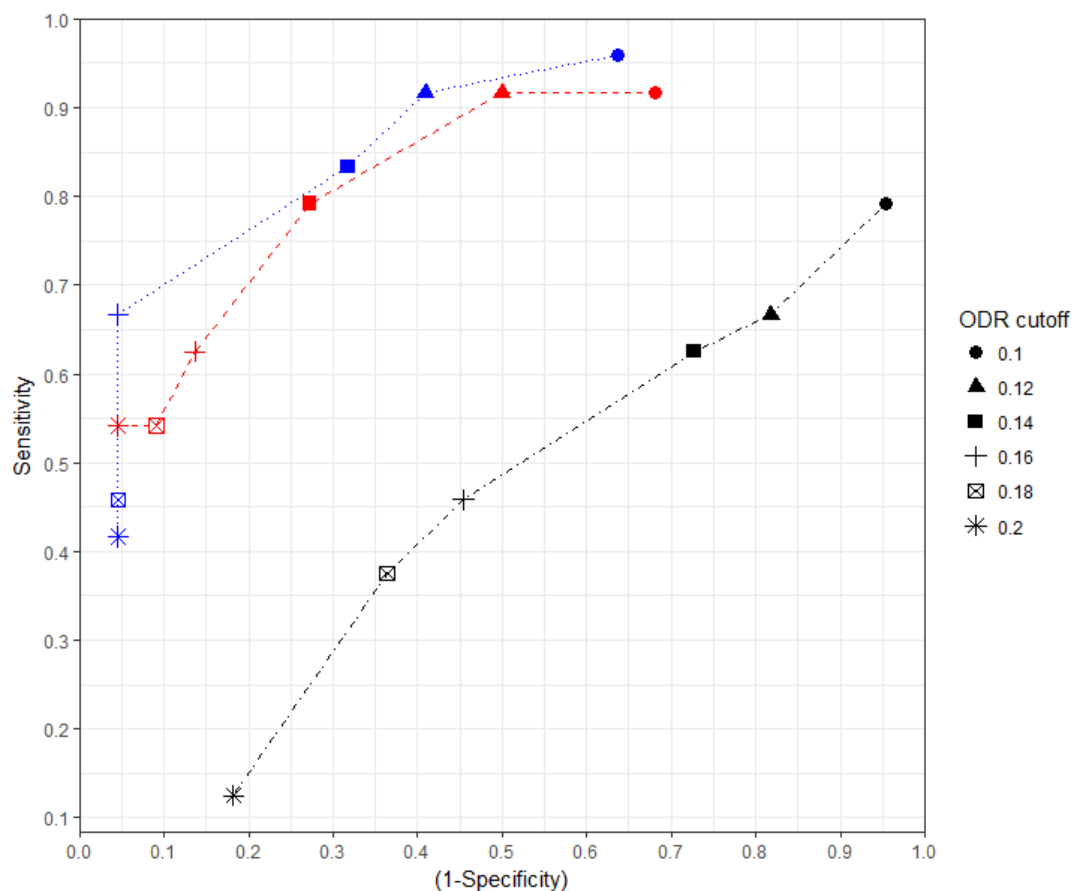


Figure 3-6 ROC curve showing results from pooled-milk and bulk tank testing. Blue (dotted line) and red (hashed line) indicate when either 10 or 6 heifer milk samples have been pooled and tested respectively (P_{10} and P_6). Black line shows the bulk tank results.

Using a cut-off of 0.16, a positive P_{10} test result is 14.7 times more likely in a positive herd than a negative one. Testing P_{10} in herds displaying clinical signs decreased the sensitivity to 50.0% with the positive likelihood ratio decreasing to 11.0 and the negative likelihood ratio increasing to 0.52.

Table 3.3 Sensitivity, specificity and likelihood ratios for the 10-heifer test (P_{10}) AUC=0.87

Cut-off	Sensitivity (%)	Specificity (%)	LR+	LR-
0.10	95.8	36.4	1.5	0.1
0.12	91.7	59.1	2.2	0.1
0.14	83.3	68.2	2.6	0.2
0.16	66.7	95.5	14.7	0.3
0.18	45.8	95.5	10.1	0.6
0.20	41.7	95.5	9.2	0.6

Table 3.4 Sensitivity, specificity and likelihood ratios for the 6-heifer test (P_6) AUC=0.85

Cut-off	Sensitivity	Specificity	LR+	LR-
0.10	91.7	31.8	1.3	0.3
0.12	91.7	50.0	1.8	0.2
0.14	79.2	72.7	2.9	0.3
0.16	62.5	86.4	4.6	0.4
0.18	54.2	90.9	6.0	0.5
0.20	54.2	95.5	11.9	0.5

Table 3.5 Sensitivity, specificity and likelihood ratios for the bulk milk tank AUC=0.45

Cut-off	Sensitivity	Specificity	LR+	LR-
0.10	79.2	4.5	0.8	4.6
0.12	66.7	18.2	0.8	1.8
0.14	62.5	27.2	0.9	1.4
0.16	45.8	54.5	1.0	1.0
0.18	37.5	63.6	1.0	1.0
0.20	12.5	81.8	0.7	1.1

3.3.4.2 Comparing the 10-heifer pooled-milk test to the bulk tank milk test

In the grazing herds with a veterinary diagnosis, the 10-heifer pooled-milk test (P_{10}) created higher ODR values than the bulk tank samples in 72.0% of herds, with mean P_{10} ODR of 0.21 (min 0.06, max 0.46, median 0.18) compared to mean bulk tank ODR of 0.12 (min 0.06, max 0.23, median 0.12) (Figure 3-7). In herds where the P_{10} ODR exceeded the bulk tank, P_{10} created an average ODR that was 0.10 higher than the bulk tank (min 0.004, max 0.45, median 0.06). In samples where the bulk tank ODR exceeded P_{10} , the bulk tank sample was higher by an average of 0.06 (min 0.002, max 0.24, median 0.06).

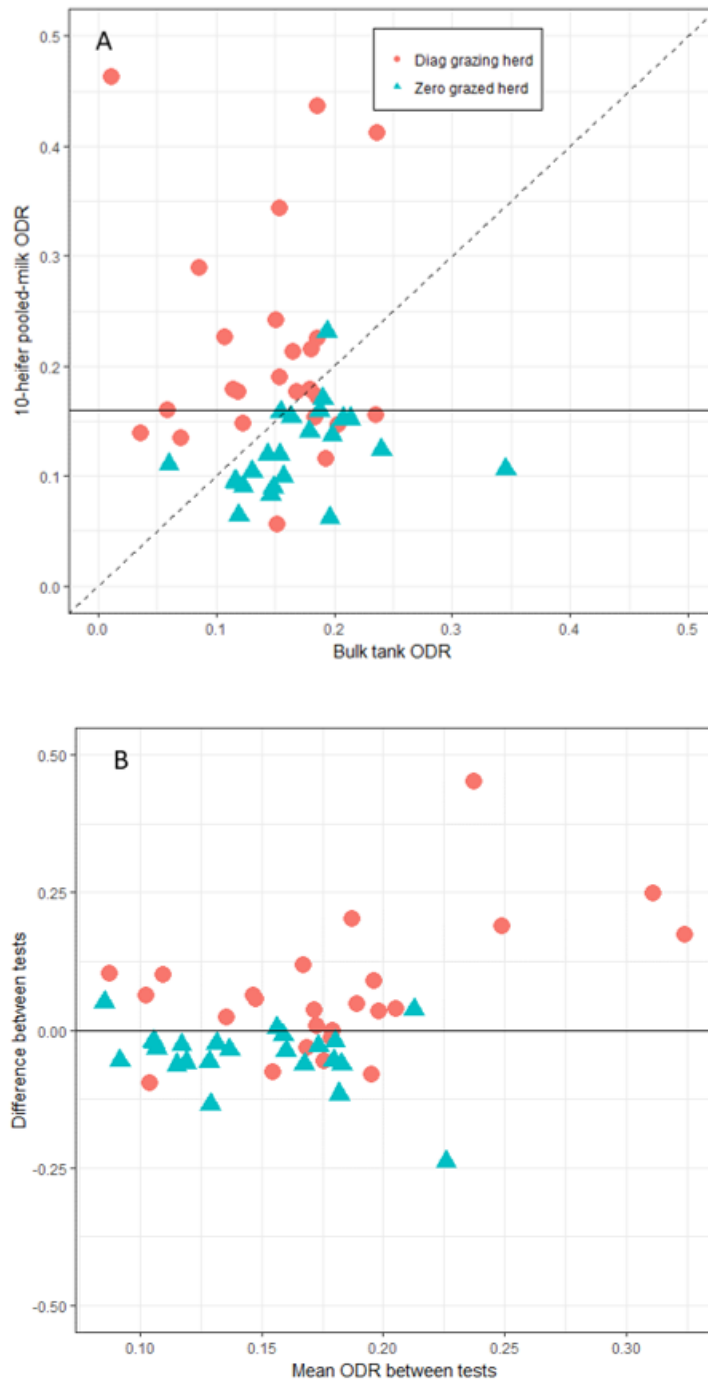


Figure 3-7 Correlation between bulk tank ODR and 10-heifer pooled-milk ODR in grazing herds with a veterinary diagnosis of lungworm (red circles) and zero grazing herds (green triangle). A) Comparison of the pooled – milk and bulk tank ODR. The diagonal line relates to the line by which $x=y$ and horizontal line the suggested positivity cut-off for the pooled-heifer test (0.16). B) Bland-Altman plot comparing difference between tests (10-heifer pooled test minus bulk tank) with mean of both tests.

3.4 Discussion

The low-sensitivity problems associated with a bulk milk test for the cattle lungworm has been well documented. This chapter describes a novel adaptation of the use of easily obtainable milk samples, using individual milk samples taken from first lactation heifers. At an ODR cut-off of 0.16, the 10-heifer pooled milk test has a sensitivity of 66.7% and specificity of 95.5%. A positive 10-heifer test result is 14.7 times more likely in a positive herd than a negative one (positive likelihood ratio). In comparison, the routinely used bulk tank milk test only provided a maximum sensitivity and specificity of 37.5% and 63.6% respectively. There was no benefit in selecting animals that were displaying clinical signs over randomly selecting animals. Clinical signs have previously been shown to be an inaccurate method of diagnosing lungworm within herds (Schnieder et al., 1993). Reasons for the low test performance in both the bulk tank and pooled heifer test could be the low numbers of positive animals within this study. Although it proved difficult to raise sensitivity to ultimately desired levels, the simple pooled heifer test presents a sharp improvement in the methodology with regards to establishing the presence or absence of *D. viviparus* on dairy farms.

A first step towards controlling lungworm is knowing whether the parasite is present on the farm. Anecdotal evidence suggests that, on many farms, the current lungworm status is unknown. Knowing whether the parasite is circulating on the farm or not, will enable the veterinarian and farmer to plan for such outbreaks and design an appropriate, targeted, evidence-based veterinary medicine approach to parasite control. In addition, the frequent purchase of animals is cited as a significant risk factor for lungworm in a multivariable model in dairy herds in Belgium (Charlier et al., 2016a). Bought-in animals will frequently be heifers. The pooled-milk test could be used to assess the lungworm status of the farm of origin, thereby assessing the risk to both the main herd and the recent arrivals. Test characteristics

described were based on results from both the autumn sampling and at turnout in May. Antibody titres were significantly higher in both heifers and cows in May, suggesting that using the pooled - milk test at turnout could be a more sensitive method of diagnosing the presence of the parasite. Moreover, testing in the autumn would provide a useful routine test to perform at the end of a grazing season. If the results suggested that the parasite was circulating on the farm, effective control measures, such as the use of the lungworm vaccination, could be planned for the following grazing season.

Other options for presence-absence testing rely on either a large proportion of the herd to have seroconverted, or clinical signs to have been witnessed. The Hannover-developed bulk tank milk ELISA, for instance, appears to offer a sensitivity of 55.6% and specificity of 92.2% but only if at least 10% of the herd have seroconverted (Ploeger et al., 2014). If the herd are showing clinical signs of the disease, at an average herd size of 73 animals (Ploeger et al., 2012), testing 6 randomly selected heifer serum samples has been shown to represent a 95% probability of detecting at least 1 positive animal.. However, if the herd needs to be tested for reasons of herd health planning, neither prior knowledge on seroconversion rates nor clinical signs will be present. The pooled-milk test can be used prior to clinical signs being present and in larger herd sizes (up to 400 lactating cattle in the present longitudinal study).

Milk samples from heifers had an ODR that was, on average, 0.05 higher than that from cows. This is in contrast to work in the Netherlands which found no significant difference in ODR values between the two age groups, despite finding a significantly higher proportion of heifer sera above the cut-off for seropositivity than older cows (26.1% heifers vs 17.5% cows) (Ploeger et al., 2012). In contrast to rises seen after primary infections, second or subsequent lungworm infections often do not stimulate measurable antibody titre rises (Strube et al.,

2017). Presumably, this is because infection of immune and immunocompetent older animals does not result in significant mature worm burdens. However, Ploeger *et al.* (2012) found that only 50% of heifers and 74.7% of cows which were excreting lungworm larvae were concurrently seropositive. This may reflect either tolerance of low-level worm infection by the host or that humoral immune responses play a limited role in lungworm control in cattle. Either way, it presents difficulties for the serodiagnosis of the parasite (which the presented work has attempted to circumnavigate). Strube *et al.* (2017) found that antibody titres were dose independent from as few as 25 lungworm larvae even in first time infections suggesting inherent host differences in antibody response to the same parasite burden. These differences may reflect genetic differences between cattle although it is also possible that in natural settings, additional unquantified factors, such as co-infection, play a confounding role in a host's immune response. For example, there is a negative inverse relationship between *Fasciola hepatica* and antibody response to *Mycobacterium bovis* as detected on the single intradermal comparative cervical tuberculin test (Claridge *et al.*, 2012). *F.hepatica* infections reduce the host's ability to produce antigen-specific T cells and therefore reduced interferon- γ (IFN- γ) production targeted against *M.bovis*. The antibody mediated Th2 response is beneficial to lungworm clearance (Kooyman *et al.*, 2002) so the role that coinfections, particularly those which enhance a cellular Th1 response, play in influencing the immune response to lungworm may need further investigation. Further work is needed to identify the heritability of antibody responses. For example, if breeding values for immune responses were available, it would be easy to select heifers to be included in the test and its sensitivity would quite likely be raised further.

The higher area under the curve for the 10-heifer pooled-milk test (0.87) compared to the bulk tank milk test (0.45) suggests that the diagnostic value of the pooled-milk test

substantially exceeds bulk tank testing. The pooled-milk test can create an ODR which is 0.45 higher than the bulk tank whereas the bulk tank only exceeds the pooled-milk test by a maximum ODR of 0.24. This may suggest that the low test performance of the bulk tank could be due to a dilution effect of a small number of antibodies in a large volume of milk. Herds where the bulk tank test exceeds the pooled-milk test will presumably have a higher proportion of positive heifers represented than in the randomly chosen pooled-milk test.

The pooled-milk test has the assumption that 10 random milk samples will be pooled. The ability to select 10 random heifers is open to human error and in reality, farmers may select animals who either have been recently coughing or showing suggestive clinical signs. The decrease in likelihood ratio in symptomatic herds implies that following clinical signs is not a reliable indicator for the disease and highlights the importance of randomly selecting heifers. It is also possible that farmers and veterinarians will not sample precisely 1ml from each animal. The role that these practical factors have on influencing the sensitivity and specificity of the test is yet to be known. Work on the number of animals to be included in composite faecal egg count tests has shown, however, that test outcome is sensitive to which animals are included but not to exactly how much of the sample of that animal is included (Presland et al., 2005) . Regardless, the presented test could function favourably as a quick, cheap, commercial test performed by milk recording companies who could be better placed to randomly sample heifer milk samples as either a stand-alone test or as part of a panel of diseases for testing on farms.

This study used data collected in one year as the basis for the bootstrap analysis on how many animals to be included in the test. Between-year differences in parasite burdens at pasture may lead to different distributions of antibody titres over the animals in the herd.

However, variability in distributions was included for four herds and the test was shown to function even when a low proportion of the animals had seroconverted. Further work could validate this. Further work is also needed to understand how the presence of the parasite on the farm relates to the risk of a clinical outbreak within a herd. The following chapter (Chapter 4) makes a start with this process.

3.5 Conclusion

In conclusion, the presented work provides a novel method of identifying the parasite within dairy herds that works in larger herd sizes in the absence of clinical signs. The 10-heifer pooled milk test has a sensitivity of 66.7% and specificity of 95.5% and is 14.7 times more likely in a positive herd than a negative one. Pooled milk tests, rather than individual milk tests, represent a cheap option for presence-absence testing. It could be used at the end of a grazing season to test for the presence of the parasite on farm and therefore to plan effective control measures, such as the use of the lungworm vaccination, for the following grazing season. It would also create a reliable option of testing cattle as they enter the herd. Further work on understanding the link between the presence-absence of lungworm on farm and the risk of a clinical outbreak is needed.

3.6 Acknowledgements

The work presented is my own. The exceptions to this are the unpublished data from a longitudinal study where dairy heifers and cows were sampled. This was kindly provided by Professor Johan Höglund in order for the sample size calculation to be performed.

Chapter 4: Associations between *Dictyocaulus viviparus* pooled milk antibody titres and farm management practices in the UK

4.1 Introduction

Parasite populations show geospatial aggregation both locally (on pasture) and nationally (Bennema et al., 2009), with parasite abundance in any particular area being driven by a mixture of farm – management and climatic variables (Nansen et al., 1978). The timing of *Nematodirus battus* eggs hatching for example, is almost entirely dependent upon daily mean temperatures (van Dijk and Morgan, 2008) yet the intensity of disease observed depends upon time of lambing and the presence of susceptible lambs at pasture when the hatch occurs (Gethings et al., 2015). Likewise, farm management practices together with the number of intermediate host (*Galba truncatula* and *Radix* spp.) habitats can predict the infection risk for *Fasciola hepatica* in cattle (Bennema et al., 2011; Charlier et al., 2011). It could be assumed, that lungworm disease (and seroconversion to *Dictyocaulus viviparus* antibodies) equally depends upon a balance between farm management and climatic variables.

Despite the inherent capability of cattle to develop a strong immunity to invading *D. viviparus* larvae (Michel, 1962; Michel and Mackenzie, 1965), the development of a successful commercial vaccine (Glaxo, 1988), and no reported resistance to any of the commonly used anthelmintics, parasitic bronchitis remains an unpredictable disease which is difficult to control (David, 1999). Regional differences in lungworm prevalence have been observed in

Belgium (Charlier et al., 2016a), Switzerland (Frey et al., 2018), Germany (Schunn et al., 2013), the Netherlands (Dank et al., 2015) and Ireland (Bloemhoff et al., 2015), suggesting that relatively subtle climatic differences may influence parasite abundance. However, whilst precipitation and temperature were initially significant predictors of bulk tank antibody levels in Germany, these variables failed to retain significance in the final model. Ultimately, only the presence of water bodies and proportion of grassed area were deemed significant factors (Schunn et al., 2013) leading the authors to argue that unmeasured farm management factors, rather than climatic factors, may be the strongest determinant of lungworm prevalence in dairy herds.

Various farm management factors have previously been associated with an increased risk of a farm outbreak. In a study investigating the causes of 25 lungworm outbreaks, 20 were thought to have occurred due to contamination by carrier animals, with the majority (n=12) being due to carrier animals having grazed the same pasture within the past 2 months (Saatkamp et al., 1994). Therefore, grazing management could be important to the risk of lungworm disease on farm. The overuse of anthelmintics has been proposed by many to prohibit natural immunity development and thereby leaves herds vulnerable to outbreaks of lungworm disease (Borgsteede et al., 1998; Ploeger et al., 2000; Vercruysse and Claerebout, 2001). Frequently purchasing animals was associated with an increased bulk tank antibody titre (Charlier et al., 2016a), which presumably reflects entry points for the parasite onto farms in a naïve herd. Herd sizes were also positively associated with antibody titre (Charlier et al., 2016a), and higher stocking densities were found to be significant in earlier studies (Oakley, 1982). However, a cross sectional study in Germany found no effect for either stocking density or size of farm (Schnieder et al., 1993).

In a study from Lower Saxony (Germany), the majority of herds who grazed for less than 150 days a year were seronegative (Schnieder et al., 1993). Mowing pasture in-between grazing cycles (for example to produce hay or silage), significantly reduced seroprevalence rates in first year grazing cattle (Schnieder et al., 1993) and bulk tank milk samples (Charlier et al., 2016a). Conversely, harrowing has been shown to transpose larvae from faeces to herbage and created a 3-fold increase in larval count in 3 days (Baxter et al., 1959).

Multivariable modelling has traditionally been used as the analysis method of choice for risk factor studies, claiming to identify significant covariates, confounders and interaction variables. However, there are a number of problems with such an approach. First, multivariable models assume a direct effect from each and all covariates in the model and as such, the calculated significance of each variable assumes a contribution from a specific covariate pattern (Lewis and McCormick, 2012). Second, in complex biological systems, it can be difficult to identify the key risk factors to apply an intervention to, in order to have both a direct and indirect effect on the outcome of interest. Third, factors that make neither biological sense nor can be manipulated may turn out to be significant in the model. Fourth, the large number of potential variables will often lead researchers to enter all variables into a single model using statistical methods, such as stepwise variable entry, rather than biological reason. The consequence of misinterpreting stepwise regression models and assuming that all variables should be considered equally is referred to as the “Table 2 fallacy” (Westreich and Greenland, 2013). Conversely, Bayesian network analysis (Heckerman et al., 1995) separates direct from indirect associations. It is this application of biological reasoning that allows for a more considered variable entry and has the potential to offer higher-level thinking regarding targeted interventions.

Directed acyclic graphs (DAGs) are causal diagrams designed to directly represent associations between variables (Greenland et al., 1999; Lewis et al., 2011; Lewis and McCormick, 2012). Causal modelling has previously been used in for example, the fields of psychology (Moffa et al., 2017) and law (Vanderweele and Staudt, 2011) . The method has begun to be introduced in veterinary epidemiology (see for example Messam *et al.*, 2008; Firestone *et al.*, 2013; Ågren *et al.*, 2017; Wolff *et al.*, 2017) but has yet to be applied to either the field of Veterinary or Human Parasitology .

In conclusion, the effects of some farm management practices on the apparent prevalence of the parasite has been demonstrated. However, it is currently unclear a) which farm management practices have the strongest effect and b) whether farm management or climatic factors, or a combination of both, determine whether disease will be seen on the farm. What is needed is a detailed study investigating the effect of a large number of farm management factors on measurements of parasite abundance. This study therefore aims to identify farm management practices which can make a herd more susceptible to a lungworm outbreak and which can be targeted for interventions in controlling the disease. The analysis compares a directed acyclic graph modelling approach to a traditional stepwise linear regression modelling technique.

4.2 Methods

This study was reviewed and approved the by University of Liverpool Veterinary Research Ethics committee (VREC 526).

4.2.1 Questionnaire development

The published literature was mined for all studies discussing farm management risk factors for subclinical or clinical lungworm disease, seroconversion to *D.viviparus* antibodies or *D.viviparus* larval faecal patency. Searches were carried out using the databases Scopus, Google Scholar and the University of Liverpool's DISCOVER service which searches all electronic and printed books, journals and theses available to access through the University of Liverpool. Search terms applied were "*dictyocaulus viviparus*" OR "*dictyocaulus*" AND "bovine" OR "cattle"; and "lungworm" AND "cattle" OR "bovine". All studies were included which looked at one or more risk factors on dairy farms. Searches were limited to the English language.

Risk factors were grouped into the following four categories: basic herd management, pasture timings, pasture management, and anthelmintic use (Table 4.1)

Table 4.1 Risk factors hypothesised to impact on the incidence of lungworm disease in dairy herds. Note that not all variables were found to be significant in the studies indicated, but were deemed important enough to consider for risk factor analysis.

Category	Variable assessed	Reference
Basic herd management	Herd enterprise (dairy vs mixed)	Bloemhoff et al., 2014, 2015
	Calving pattern	Bloemhoff et al., 2014, 2015
	Number of adult cows	Charlier et al., 2016
	Frequent purchase of animals	Charlier et al., 2016
	Supplemental feeding	Schnieder et al., 1993 Höglund et al., 2004
Pasture timings	Turnout cows	Schnieder et al., 1993 Bloemhoff et al., 2014, 2015
	Housing cows	Bloemhoff et al., 2014, 2015
	Grazing length cows	Schnieder et al., 1993 Bloemhoff et al., 2014, 2015
Pasture management	Pasture rotation(permanent vs rotational)	Bloemhoff et al., 2014
	Stocking rate	Schunn et al., 2013 Bloemhoff et al., 2014
	Move onto silage/hay aftermath	Höglund et al., 2004 Bloemhoff et al., 2014
	Slurry spread on pasture to be grazed	Bloemhoff et al., 2014
	Rearing of young stock (home bred or contract reared)	Bloemhoff et al., 2014
	Leader follower system used	Bloemhoff et al., 2014
	Pasture mowed prior to grazing	Schnieder et al., 1993 Charlier et al., 2016
	Dose and move used	Bloemhoff et al., 2014, 2015
	Whether pastures were rested between grazing seasons	Höglund et al., 2004
	Mixed age grazing	Schnieder et al., 1993 Höglund et al., 2004
	Presence of lentic water bodies	Schunn et al., 2013
Anthelmintic treatment	Cows	Bloemhoff et al., 2014, 2015
	In calf heifers	Bloemhoff et al., 2014, 2015
	Prophylactically vs when see clinical signs	Höglund et al., 2004
	Anthelmintic category	Ploeger et al., 2000

Based on the literature search, a questionnaire was developed consisting of 35 questions in six sections (Appendix 2). The first comprised a demographic section to determine the farm name and location. This was necessary in order to collect the corresponding milk samples from the milk recording company but it was made clear that anonymisation would occur prior to data analysis and results becoming published. The second section asked questions about the general farm structure including the number of calves, first lactation milking heifers (hereafter 'heifers') and adult cattle (hereafter 'cows') in the herd; the presence and number of beef cattle on the farm; the method for replacing cattle on farm (breeding own replacements or buying new cattle); the number of dairy cattle (calves, first lactation milking heifers and adult cattle) bought onto the farm during 2016; calving pattern (spring, autumn or all year round); whether they were or had ever been classed as an organic farm; dates of turnout for calves, heifers, cows and dry cows in 2017; and planned date of housing for all age groups in 2017.

The third section asked about contact between cattle. Questions were asked as to whether their cattle had contact with any other farm's cattle, through for example, a shared fence or shared grazing. Respondents were asked whether pasture grazed by their cattle had a permanent water body (defined as a pond, stream or river) as this has previously been defined as one of the only significant risk factors for *D.viviparus* in Germany (Schunn et al., 2013). Respondents were asked a series of true or false questions on whether each age group (calves, heifers or adult cows) would routinely be put onto pasture that had been grazed by another age group without resting the pasture for at least two months between age groups. Two months was selected as the minimum resting period because when pasture was rested for six weeks, tracer calves had no lungworms present at necropsy but small numbers of larvae were still found on pasture (Eysker et al., 1992). Furthermore, in a study looking at the

causes of lungworm outbreaks, the majority of cases were caused by carrier animals grazing the same pasture within two months (Saatkamp et al., 1994). Finally, respondents were asked whether any age group of cattle grazed away from the business address for any length of time (e.g. contract – reared calves). Respondents were then asked which age group grazed away from site in an open – ended question.

The fourth section asked questions pertaining to clinical signs or history of lungworm disease on the farm. Respondents were asked closed questions on whether they had seen persistent coughing or a lungworm outbreak in different age groups of animals at different time frames (during the current grazing season, last grazing season, two years ago, three to five years ago, longer than five years ago or never). Persistent coughing was defined as a cough that happened on more than one occasion. Outbreaks were defined as a significant milk drop, clinical signs in several animals or mortalities. They were asked whether they had ever received a veterinary diagnosis of lungworm in each age group and time frame. Each diagnostic method was assessed separately: dung test, blood test, individual milk test, bulk tank milk test and post – mortem.

The fifth section asked questions regarding the lungworm vaccination use including whether they used the vaccine, how soon after giving the second dose of vaccine they turned the cattle out and which age group they gave the vaccination to. The sixth section asked questions regarding anthelmintic treatments. Respondents were asked to select months over the preceding 12 months where anthelmintics had been given to each age group and to name the anthelmintics applied. In addition, questions were asked regarding whether a quarantine protocol was in place for cattle entering the herd and whether this involved using anthelmintics.

In addition, respondents were asked two open-ended, qualitative questions. The first was “Do you think that you have lungworm on your farm?” This question was asked to assess the degree of accuracy and farmer knowledge of lungworm on the farm. The second question was “Why do you, or don’t you, use Huskvac [the lungworm vaccine]?” This was asked as a simple, initial exploration into the motivations behind vaccine use.

4.2.2 Study population and recruitment

UK based dairy herds in the Tesco Sustainable Dairy Group (TSDG) were included in the study. This group were approached because of their broad geographical distribution within the UK and effective communication between the TSDG management and individual farmers. Only herds where the adult cattle grazed pasture for at least 40 days from April to October (n=652) were eligible for inclusion in the study. A minimum of 40 grazing days was selected in order to allow time for at least one full lifecycle of the parasite to develop on pasture (Eysker, *et al.*, 1994). The TSDG group contains dairy farmers who have contracts with two major milk buyers, Arla Foods UK (31.3% grazing farms in the TSDG group, n=204) and Müller UK and Ireland (68.7% grazing farms in the TSDG group, n=448). From the TSDG grazing herds, the proportion in each region of Great Britain are as follows: midlands 31.7% (n=207), southwest England 25.5% (n=166), northwest England 19.9% (n=130), Scotland 9.0% (n=59), Wales 9.0% (n=59), southeast England 4.8% (n=31).

A postal questionnaire was sent out on 30th May 2017 to all eligible TSDG farmers together with a self – addressed freepost envelope. Prior to sending the questionnaire out, it was tested amongst veterinary surgeons and managerial staff within the TSDG group which included several dairy farmers. The postal questionnaire also provided a link to an online version of the same questionnaire and farmers were given a choice on how to fill it in. The

online questionnaire closed, and farmers were asked to return postal questionnaires by the 11th August 2017 (10 weeks later) to allow processing and collection of milk samples in the autumn. Monthly reminders were sent out via email and text message from the TSDG management team.

4.2.3 Milk sample collection and testing

During October and November 2017, an individual milk sample were collected from milk processors from each milking heifer in their first lactation (hereafter, “heifers”), from all participants who completed the questionnaire. Ten heifer milk samples were then randomly selected per herd using a random number generator. Pooled milk samples were produced by mixing 1ml milk sample from each of the 10 heifer samples as described in Chapter 3. This was tested using a prototype of an ELISA plate developed by Boehringer-Ingelheim Svanova (Uppsala) with a positivity cut-off for the pooled-milk test of 0.16. Immediately after collection, milk samples were pooled, defatted by centrifuging at 2,000g for 15 minutes and separating the supernatant from the lipid layer, and stored at -20°C until tested. All samples were tested within 30 days of freezing. Responses from herds where pooled-milk samples were unavailable were excluded from analysis.

4.2.4 Data analysis

Hard-copies of the questionnaire were entered into an online questionnaire development software (SurveyMonkey, www.surveymonkey.com, Portland, Oregon, USA) and checked for data entry errors. All data analysis was performed in the R statistical program (RCoreTeam, 2017).

The length of the grazing season for each age group was calculated as a numerical variable using the number of days between the date of turnout to the date of housing. If respondents completed the month and year for turnout or housing but not the day, the date was assumed to be the 14th of the month. Exceptions to this rule were if farmers commented that the date was “early”, in which case the 7th of the month was chosen, or “late” where the 21st was assumed. Postcode data was converted into Nomenclature of Territorial Units for Statistics (NUTS) region 1 data (Office Of National Statistics, 2018).

The results of the pooled – milk ODR were tested for normality by plotting density plots, quantile – quantile (QQ) plots and using the Shapiro – Wilk test. The prevalence in each region (NUTS 1 category) were calculated only if there were more than five respondents per region (in order to maintain anonymity). The pooled – milk ODR was compared between regions using the Kruskal Wallis test. Variables were described as means and 95% confidence intervals for continuous, normally distributed data; medians and interquartile range for non-parametric continuous data; and counts and percentages for categorical data. The sensitivity and specificity of detecting coughing or farmer’s thinking that they had lungworm on the farm was calculated as per Thrusfield (2007). True positives were classed as farmers who reported coughing / thought they had lungworm and had a pooled milk test $ODR \geq 0.16$. True negatives were classed as the number of farmers who reported that they had not seen coughing / thought that lungworm was not on the farm and had a pooled milk test $ODR < 0.16$.

Sensitivity was then calculated as:

$$Sensitivity = \frac{True\ positives}{Number\ of\ farms\ with\ pooled\ milk\ ODR \geq 0.16} \quad Equation\ 3$$

Specificity was calculated as:

$$Specificity = \frac{True\ negatives}{Number\ of\ farms\ with\ pooled\ milk\ ODR < 0.16} \quad Equation\ 4$$

Variables were assessed for collinearity using Pearson's correlation and key variables were assessed for the proportion of missing data. Where the number of missing continuous data was large, variables were converted into categorical data if possible. Variables were excluded only if there was logical reason to do so.

4.2.5 Directed acyclic graph modelling approach

A master directed acyclic graph (DAG) was developed which represented all the direct and indirect associations between risk factors for lungworm titre in dairy herds. This was based on a combination of literature searching and answers from the questionnaire. A causal relationship between covariates was assumed if an intervention on a parent node would affect the values of the child node (Vanderweele and Staudt, 2011).

One key strength in using DAGs to assess causation lies in its ability to identify both direct and indirect effects through the use of "backdoor paths" (Pearl, 1995). For each exposure of interest (X), a list of backdoor paths was created which listed all covariates that begin with an arrow into X and continues in a cyclic path until reaching the outcome, in this case,

lungworm titre. Confounding in the DAG approach occurs only if backdoor paths are identified, unlike in the traditional stepwise regression modelling where every factor is judged to be a “confounder” (Suttorp et al., 2015). For each exposure, the minimum number of necessary covariates which would block all backdoor paths was identified (Pearl, 1995). Multivariable linear regression models were created for each exposure of interest and their unique set of necessary covariates. If no backdoor paths were identified between an exposure and the outcome then simple linear regression was used.

4.2.6 Stepwise linear regression modelling approach

For the traditional stepwise modelling approach, simple linear regression models were used for all exposures to assess the relationships with pooled – milk antibody titres. Variables where $p \leq 0.30$ were taken into a multivariable linear regression model. Models were fitted using a manual backward stepwise elimination approach with re-entry and testing of model fit using the likelihood ratio test with the aim of minimising the Akaike information criterion (AIC) values. Finally, region was entered as a random effect into a mixed effect model to see if this improved the model fit.

4.3 Results

Out of 652 grazing TSDG farmers approached, 147 farmers returned the questionnaire and 92 also had pooled milk samples available for testing (combined questionnaire and sample response rate 14.11%). The pooled - milk ODR deviated from showed a non – parametric distribution ($W=0.91$, $p<0.001$) (Figure 4-1). The median pooled - milk ODR was 0.17 (25th - 75th quantile: 0.14, 0.21)

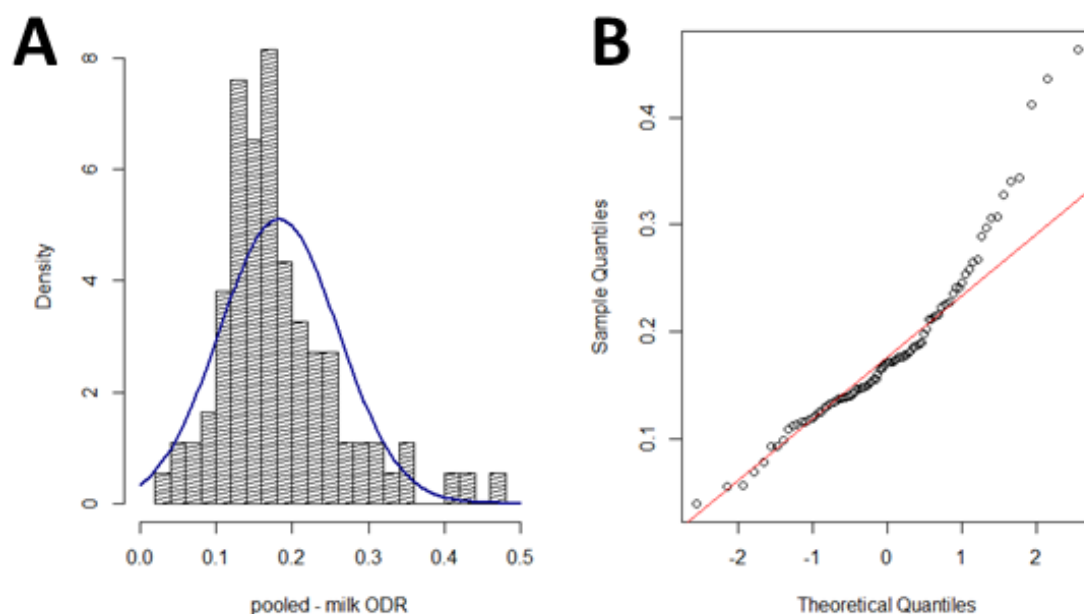


Figure 4-1 Density plot (A) of pooled – milk antibody titre (ODR) from 10 heifers in each herd in the cross – sectional study. Normal curve is superimposed. B) Quantile – quantile (QQ) plot showing deviation from linearity representing distribution that is non – normal.

4.3.1 Descriptive data

4.3.1.1 Farm demographics

Out of the 92 participating farms, 31.5% milk recorded with the Cattle Information Service ($n=29$), 59.8% with National Milk Records ($n=55$) and 8.7% with Quality Milk Management Services Ltd ($n=8$). The geographical distribution of the study farms (Figure 4-2) closely

follows the cattle density maps for Great Britain (DEFRA, 2008). Amongst NUTS 1 regions where more than five study farms were included, the highest prevalence of positive dairy herds occurred in Wales (61.5%, 8 positive out of 13 herds), followed by southwest England (59.1%, 13 positive out of 22 herds), the west midlands (55.2%, 16 positive out of 29 herds) and north west England (50.0%, 16 positive out of 29 herds). Scotland had the highest median ODR at 0.2 although there were no significant differences between regions ($H(7)=9.22$, $p=0.24$) (Figure 4-3).

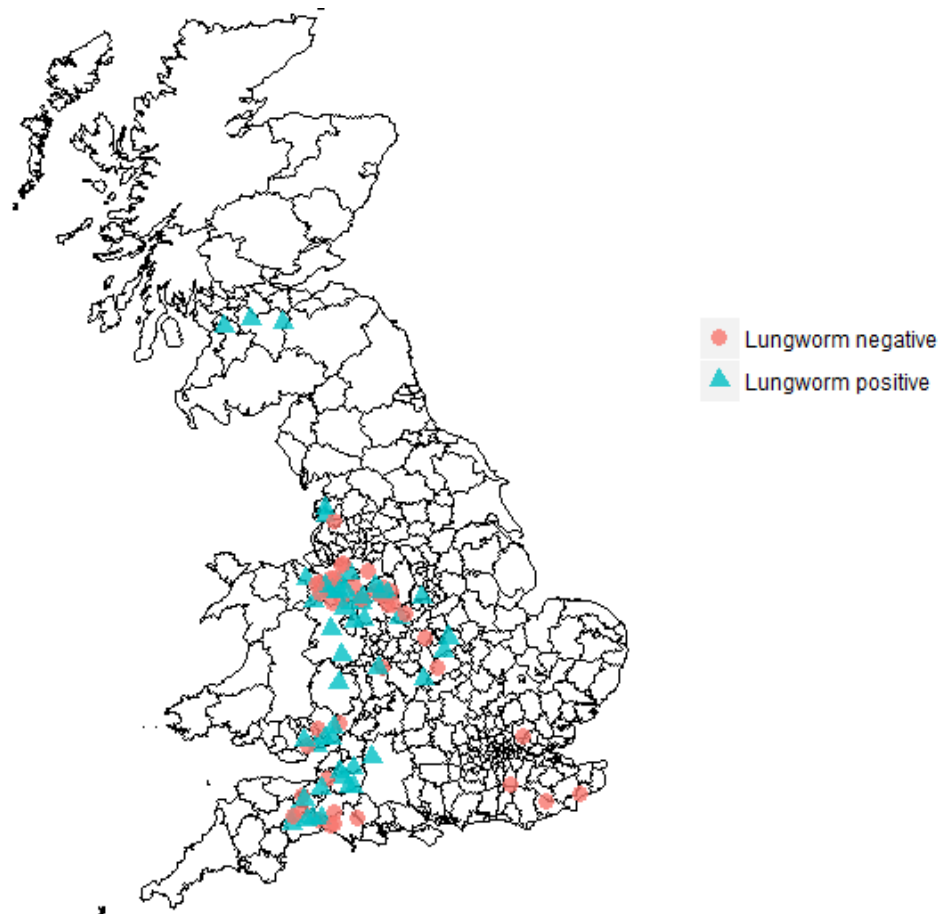


Figure 4-2 Geographical distribution of study farms across Great Britain showing positive herds in green triangles and negative herds in red circles.

4.3.1.2 General farm structure

The average herd size (median 278 cattle, 25th - 75th quantile: 198, 444) consisted of 185 cows (25th - 75th quantile: 116, 278), 70 heifers (25th - 75th quantile: 44, 108) and 40 calves (25th - 75th quantile: 20, 71). Additionally, 46.7% of farms kept beef animals, average beef herd size 32 (25th - 75th quantile: 10, 78). In order to replace cattle on the farm, 68.5% bred their own replacement cattle, 7.6% bought cattle onto the farm and 21.7% herds bred replacement cattle and bought cattle onto the farm.

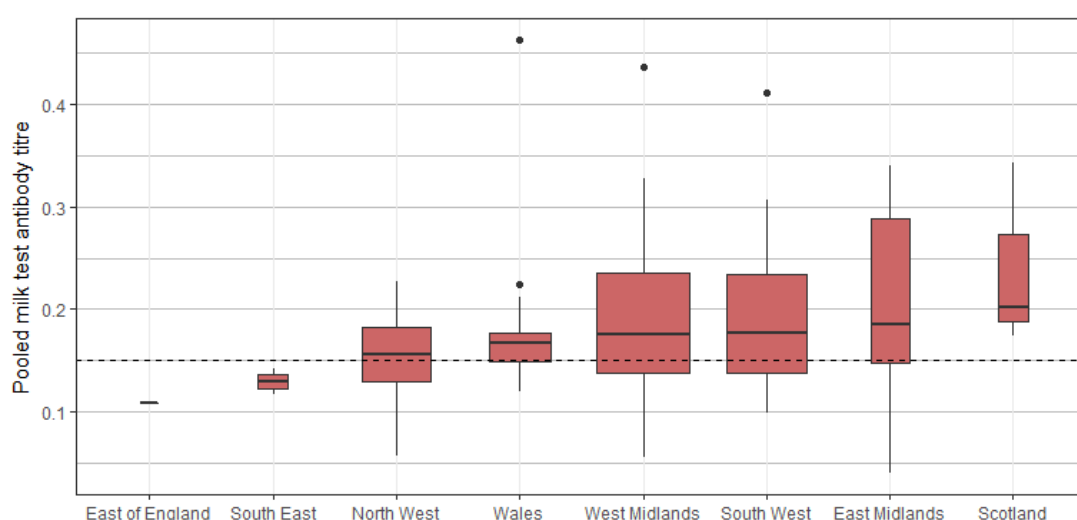


Figure 4-3 Boxplot showing distributions of pooled milk *Dictyocaulus viviparus* antibody titres by NUTS 1 region. Width of boxplot is proportional to the number of study farms in each location. Horizontal hashed line refers to pooled – milk ODR positivity cut off of 0.16

In the preceding year, 48 farmers did not bring any calves onto the farm, 43 did not bring any heifers and 43 did not bring any cows onto the farm. Out of respondents who did bring cattle onto the farm, an average of 3 calves (25th - 75th quantile: 3, 20), 15 heifers (25th - 75th quantile: 9, 39) and 10 cows (25th - 75th quantile: 5, 26) were bought in. The majority of respondents calved all year round (85.9%, n=79). No herds were currently, nor had ever been, classed as organic. The average date of turnout for calves, heifers and cows was the 9th May,

17th April and 8th April respectively with the average date of housing on the 15th October, 2nd November and 15th October. Calves grazed for an average of 153.8 days during the summer (95% confidence intervals 53.5 - 327.3), heifers grazed for 201.8 days (95% confidence intervals 150.4 - 276.1) and cows for 192.3 days (95% confidence intervals 134.6 - 263.6).

4.3.1.3 Contact between cattle

Cattle on 34 farms (34.1% participants) had access to cattle from another farm through a shared fence. The majority of farms (70.7%, n=65) had a water body on site (classed as a stream, pond or other water source). The majority (75%) of farmers did not adopt a follower grazing system at pasture. Only 14.1% calves, 15.2% heifers and 10.9% cows used pasture that had been grazed by a different age group within the past two months. The majority of herds (52.2%, n=48) grazed cattle away from the main herd. Farmers would most frequently graze prepubescent and in-calf heifers away from the main herd (89.8% farms, n=44) or dry cows in 20.4% (n=10) farms.

4.3.1.4 Lungworm history

A total of 25% farmers (n=23) reported seeing coughing in either their cows, heifers or calves over the preceding 12 months. The sensitivity and specificity of farmer's reporting coughing compared to the pooled milk test was 23.5% and 73.2% respectively. Out of responding farmers, one farm had seen a serious lungworm outbreak during the present grazing season, four farms saw an outbreak during the preceding year, two had witnessed an outbreak two years ago, five had an outbreak between three and five years ago and thirteen had seen an outbreak more than five years ago. A total of 65.3% farmers (n=47) reported that they had never seen a serious lungworm outbreak. The majority of cases were diagnosed through

faecal sampling (31.8%, n=7 farms) followed by bulk tank milk testing (27.3%, n=6), post - mortem tests (18.2%, n=4), clinical signs (13.6%, n=3) and serum sampling (9.1%, n=2).

4.3.1.5 Lungworm vaccination

The majority (62%, n=57) of farms did not use the lungworm vaccine to immunize their cattle. For farms which did use the vaccine, the majority turned cattle out between two and four weeks after giving the second dose of the vaccine (62.5%, n=20), whereas 18.8% farms turned their cattle out between four and eight weeks later (n=6), 9.4% turned out between one and two weeks later (n=3), and 3.1% turned out more than eight weeks later (n=1). In addition, 6.3% respondents vaccinated cattle which were already grazing (n=2).

4.3.1.6 Anthelmintic treatments

Anthelmintics were not administered to 15.2% calves, 22.8% heifers and 1.1% cows (excluding dry cows) (Figure 4-4). Anthelmintics were given more than twice a year to 40.2% calves, 53.3% heifers and 29.3% cows. Only 13.5% farmers (n=7) reported having a quarantine protocol in place for incoming cattle of which all farmers reported using eprinomectin as part of this protocol.

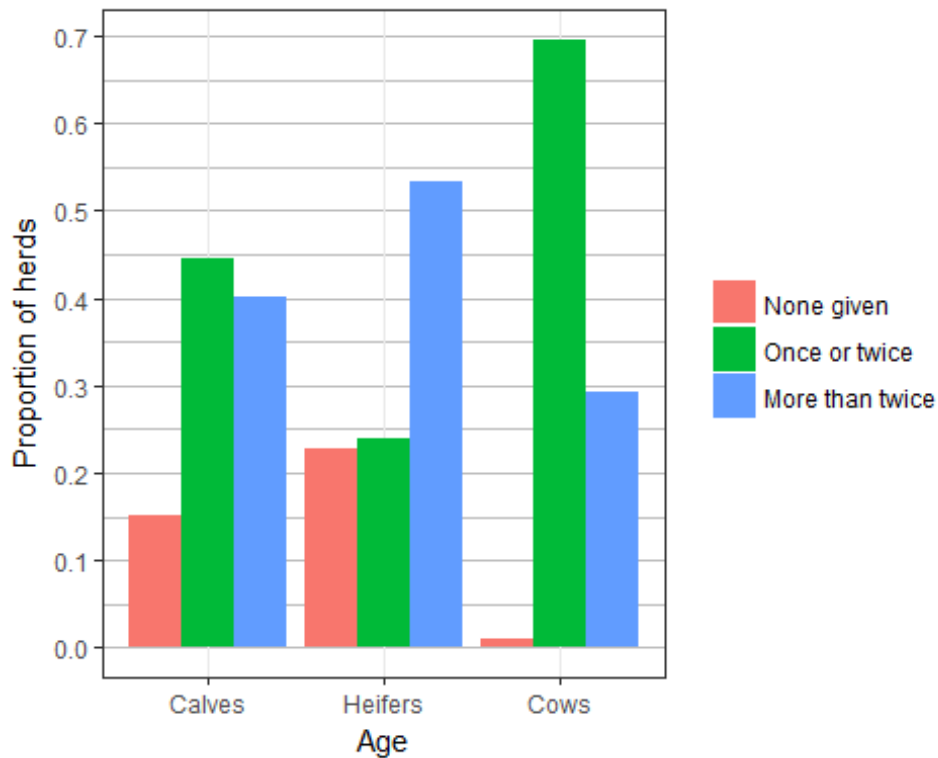


Figure 4-4 Frequency of anthelmintic dosing to grazing calves, heifers and cows in UK dairy herds.

4.3.1.7 Farmer attitudes

When asked whether participants felt that they had lungworm on the farm, 36.4% thought that they did, 34.1% thought that they did not and 29.5% did not know whether they had lungworm on the farm. According to the pooled milk testing, the sensitivity and specificity of a farmer's impression on whether they had lungworm on the farm was 45.9% and 40% respectively. Out of the responses where the farmer had indicated that they were not sure whether they had lungworm, 50% were positive for lungworm on the pooled milk testing.

Questionnaire responses to the open-ended question "why do you, or do you not, use the lungworm vaccine (huskvac)?" related to either a) an innate lack of willingness or awareness

to use the vaccine, such as "we don't think we need to" ; "we have never discussed this with the vet" or "we don't know anything about it" (n=10); or b) anthelmintics being used as an alternative, such as "regularly wormed" or "use bolus instead" (n=5); or c) logistical reasons with the length of the grazing season, for example, "we cannot get cattle out quick enough to be effective" (n=2).

4.3.2 Correlated variables and missing data

The number of heifers and cows within the herd were found to be strongly correlated ($r=0.88$, $p<0.001$), as were the number of heifers and calves ($r=0.78$, $p<0.001$) and calves and cows ($r=0.92$, $p<0.001$). Therefore, the total number of dairy cattle was combined into one variable, herd size. There were many missing answers for the number of calves (missing 39 responses), heifers (missing 31 responses) and cows (missing 30 responses) bought onto the farm in the preceding year. Therefore, herds were classed according to their cattle replacement system as a categorical variable (those which bought animals onto a farm, those who bred their own replacements and those that did both). Responses for individual anthelmintic drugs were varied and so herds were classed according to their anthelmintic dosing frequency as those that gave anthelmintics more than twice; once or twice; or never, to adult cows or calves. Herds were classed as to whether they did or did not use the lungworm vaccine. The question of whether herds quarantined animals as they came onto the farm was not analysed further because this only referred to farms where cattle were bought onto the farm. Herds were classed as those that did or did not keep beef animals rather than having the number of beef animals as a continuous variable.

The number of missing answers can be seen in Figure 4-5.

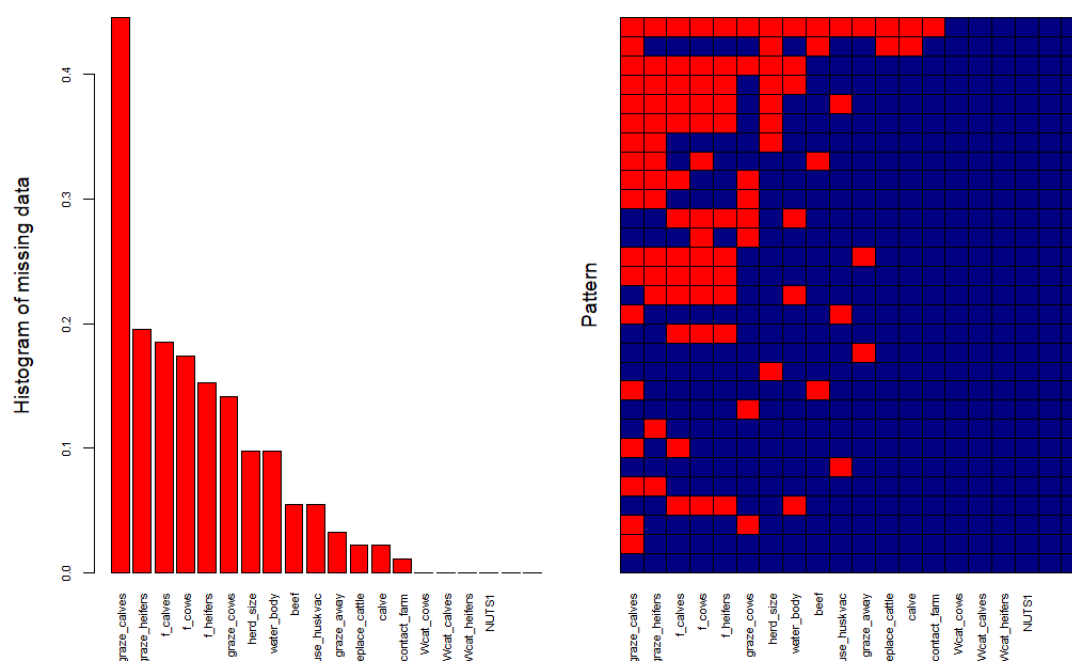


Figure 4-5 Histogram (A) and heatmap (B) of number of missing answers from cross – sectional survey of UK dairy herds. Rows of the heatmap refer to individual responses and red colours denote missing answers. Variables tested were the grazing length for calves (graze_calves), heifers (graze_heifers) and cows (graze_cows); whether the calves, cows and heifers grazed pasture which had not been rested for more than two months (f_calves, f_cows, f_heifers respectively); herd size (herd_size), presence of a water body (water_body), presence of beef animals (beef), whether the lungworm vaccine was used (use_huskvac), whether cattle graze away from the main herd (graze_away), cattle replacement system (replace_cattle), whether there is contact with any other farm (contact_farm), frequency of worming in calves, heifers and cows (Wcat_calves, Wcat_heifers, Wcat_cows) and region of the United Kingdom (NUTS1).

Five variables (grazing length of heifers and calves and whether calves, heifers or cows grazed on pasture that had been grazed by a different age group within the past two months) had more than 15% missing responses and so were excluded from further analysis. Once these variables were excluded, a total of 64.1% of responses had no missing data.

Therefore, this process identified the following key variables to take into further analysis:

- Region (NUTS1, categorical variable)
- Herd size (continuous variable)
- Presence of beef animals (dichotomised variable)
- Cattle replacement system (categorical variable)
- Calving pattern (categorical variable)
- Length grazing season for cows in days (continuous variable)
- Contact with another farm such as over a shared fence (dichotomised variable)
- Presence of a water body (dichotomised variable)
- Grazing cattle away from the main herd (dichotomised variable)
- Use of the lungworm vaccine (dichotomised variable)
- Anthelmintic frequency in cows (ordinal variable)
- Anthelmintic frequency in calves (ordinal variable)

4.3.3 Directed acyclic graph modelling approach

The master directed acyclic graph for lungworm disease in dairy herds can be seen in Figure 4-6 and Table 4.2. Several factors were identified as having a potential direct effect on the causation of lungworm antibody levels but were not thought to have any indirect associations with any of the other variables. These were: the presence of beef animals; grazing cattle away from the main herd; the cattle replacement system; the presence of a water body; contact with another farm (such as over a fence) and the herd size. After analysing the reasons why participants would chose to use or not use the lungworm vaccine, it was decided that calving pattern, anthelmintic use and length of the grazing season would all influence whether the vaccination was used. Regional differences in lungworm prevalence rates were described in Chapter 2. It was deemed probable that due to differences in climate,

there would be regional differences in the length of the grazing season. It was also thought that calving pattern and length of the grazing season would influence the anthelmintic dosing frequency.

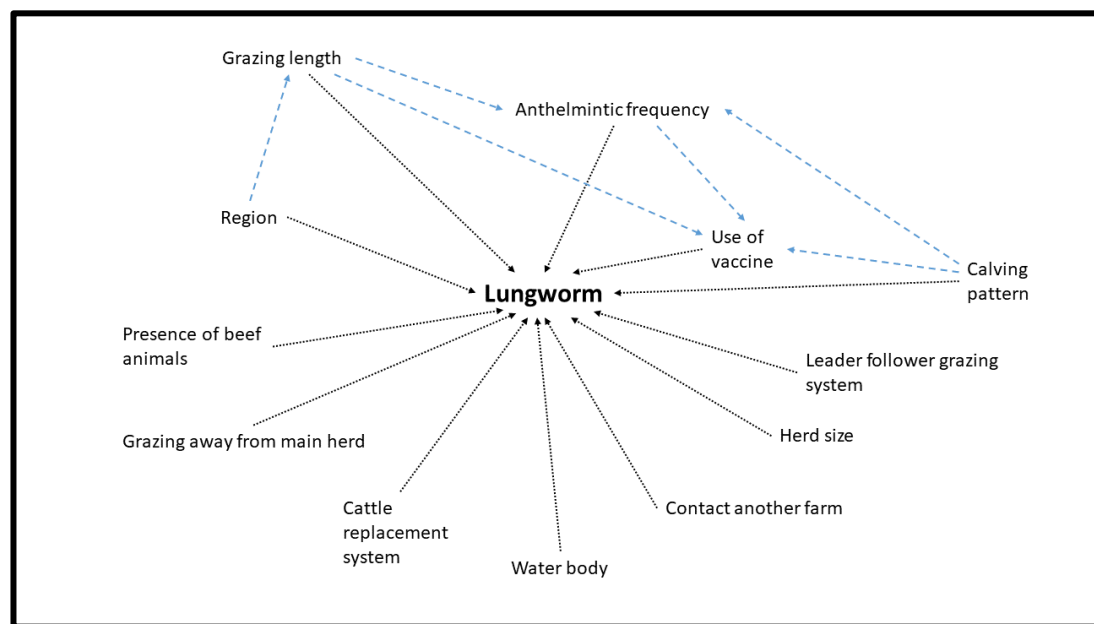


Figure 4-6 Master directed acyclic graph for lungworm disease in dairy herds. Associations were decided based on literature searching and answers from the questionnaire (Table 4.2). Black dotted lines represent potential direct associations between the exposure of interest and lungworm antibody titres whereas blue hashed lines represent directional associations between pairs of variables.

The necessary covariates between anthelmintic frequency and lungworm titre were the length of the grazing season and calving pattern (Table 4.3). Necessary covariates between the vaccine and lungworm titre were anthelmintic frequency, length of the grazing season and calving pattern. Region was the only necessary covariate for length of the grazing season.

Table 4.2 Sources of information for covariates in master directed acyclic graph for lungworm disease in dairy herds (Figure 4-6). Note that not all variables were found to be significant in the studies indicated but were deemed important enough to consider for risk factor analysis.

Factor	Source of knowledge
<i>Direct associations with lungworm antibody titre</i>	
Region	Veterinary Investigation Diagnosis Analysis database (see Chapter 2)
Herd size	Schnieder et al., 1993; Charlier et al., 2016
Presence of beef animals	Bloemhoff et al., 2014, 2015
Cattle replacement system	Charlier et al., 2016
Calving pattern	Forbes, 2016
Length grazing season	Ploeger et al., 1990; Schnieder et al., 1993; Bloemhoff et al., 2015; Charlier et al., 2016
Contact with another farm	-
Presence of a water body	Schunn et al., 2013
Grazing away from the main herd	David, 1999
Use of lungworm vaccine	Ploeger et al., 1990
Anthelmintic frequency	Ploeger et al., 1990; Eysker et al., 1995; Bloemhoff et al., 2015; Charlier et al., 2016
<i>Indirect associations between variables</i>	
Region -> Length grazing season	-
Length grazing season -> Anthelmintic frequency	-
Length grazing season -> Use of vaccine	Questionnaire responses
Anthelmintic frequency -> Use of vaccine	Questionnaire responses
Calving pattern -> Use of vaccine	Questionnaire responses
Calving pattern -> Anthelmintic frequency	-

According to the DAG modelling approach, grazing cattle away from the milking herd led to an increased pooled milk titre of 0.04 (95% confidence interval 0.01 – 0.08, $p=0.01$) (Table 4.4). The presence of a water body was associated with an increased antibody titre of 0.04 (95% confidence intervals 0 – 0.08, $p=0.05$). No other variables retained significance in their models.

Table 4.3 Backdoor paths on the causal pathway between anthelmintic use, the use of the lungworm vaccine and the length of the grazing season with the lungworm antibody titre. Note that the minimum number of necessary covariates in all backdoor paths are underlined.

Exposure	Backdoor paths
Anthelmintic frequency	<u>Length grazing</u> <u>Length grazing</u> : Region <u>Length grazing</u> : Vaccine <u>Calving pattern</u> <u>Calving pattern</u> : Vaccine
<i>Lungworm titre ~ $\beta_{anthelmintic\ frequency} + \beta_{length\ grazing\ season} + \beta_{calving\ pattern}$</i>	
Vaccine	<u>Length grazing</u> <u>Length grazing</u> : <u>Anthelmintic frequency</u> <u>Length grazing</u> : Region <u>Anthelmintic frequency</u> <u>Anthelmintic frequency</u> : <u>Length grazing</u> <u>Anthelmintic frequency</u> : <u>Length grazing</u> : Region <u>Anthelmintic frequency</u> : <u>Calving pattern</u> <u>Calving pattern</u> <u>Calving pattern</u> : <u>Anthelmintic frequency</u>
<i>Lungworm titre ~ $\beta_{vaccine} + \beta_{anthelmintic\ frequency} + \beta_{length\ grazing\ season} + \beta_{calving\ pattern}$</i>	
Length grazing	<u>Region</u>
<i>Lungworm titre ~ $\beta_{length\ grazing\ season} + \beta_{region}$</i>	

Table 4.4 Associations between exposure variables and pooled milk *Dictyocaulus viviparus* antibody titres using a directed acyclic graph approach. For each model, only the results of the exposure variable of interest is shown. Other variables (necessary covariates), if included, are for control of potential confounding identified through directed acyclic graphs. Note that for simplicity, if categorical variables are insignificant then variable estimates for each category are not shown.

Model	Exposure variables	Necessary covariates on causal pathway	Model fit			Variable estimates			
			F	DF	P value	B (95% CI)	Standard error	P value	Adjusted R ²
1	Region	None	1.01	7, 84	0.43	-	-	-	-
2	Herd size	None	0.26	1, 81	0.61	-1.1 x 10 ⁻⁵	2 x 10 ⁻⁵	0.61	-0.01
3	Presence of beef animals	None	2.03	1, 85	0.16	0.02	0.02	0.16	0.01
4	Replacement cattle system	None	0.05	2, 87	0.95	-	-	-	-
5	Calving pattern	None	1.09	3, 86	0.36	-	-	-	-
6	Grazing length	Region	0.97	7, 71	0.46	1 x 10 ⁻⁴	3 x 10 ⁻⁴	0.59	-0.003
7	Contact with another farm	None	1.95	1, 89	0.17	0.02	0.02	0.17	0.01
8	Presence of a water body	None	3.92	1, 81	0.05	0.04 (0 – 0.08)	0.02	0.05*	0.03
9	Grazing cattle away from the main herd	None	7.50	1, 87	0.01	0.04 (0.01 – 0.08)	0.02	0.01**	0.07
10	Use of the vaccine	Anthelmintic frequency calves, grazing length, calving pattern	0.76	7, 66	0.63	-8 x 10 ⁻³	2.1 x 10 ⁻²	0.71	-0.02
11	Anthelmintic frequency cows	Grazing length, calving pattern	0.55	6, 71	0.77	-	-	-	-
12	Anthelmintic frequency calves	Grazing length, calving pattern	1.07	6, 71	0.39	-	-	-	-

Significance: * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

4.3.4 Stepwise linear regression modelling approach

Results from the simple linear regression modelling for all variables are shown in Table 4.5. The following variables had a p value below 0.3 in univariate analysis and so were retained for multivariable linear regression modelling: presence of beef cattle on the farm, contact with another farm, presence of a water body, grazing away from the mainstay and anthelmintic frequency in calves. Full details of the final multivariable linear regression model ($F(5, 71)=3.5$, $p=0.01$, $R_2=0.20$, adjusted $R_2=0.14$) can be found in Table 4.6. Results from the multivariable model suggest that grazing cattle away from the mainstay significantly increased the antibody titres by 0.04 (95% confidence intervals 0.00 – 0.07, $p=0.04$). The presence of a water body increased antibody titres by 0.04 (95% confidence intervals 0.00 – 0.08, $p=0.06$) with borderline significance in the model ($p=0.06$). No other variables retained significance in the multivariable model. The model fit was not improved when a mixed effects model was created adding a random intercept for region ($\chi^2(1)=0.26$, $p=0.61$).

Table 4.5 Simple linear regression results for variables of interest and lungworm antibody titres.

Exposure variables	Model fit			Variable estimates			
	F	DF	P value	B (95% CI)	Standard error	P value	Adjusted R2
Region	1.01	7, 84	0.43	-	-	-	-
Herd size	0.26	1, 81	0.61	-1.1×10^{-5}	2×10^{-5}	0.61	-0.01
Presence of beef animals [‡]	2.03	1, 85	0.16	0.02	0.02	0.16	0.01
Replacement cattle system	0.05	2, 87	0.95	-	-	-	-
Calving pattern	1.09	3, 86	0.36	-	-	-	-
Grazing length	0.97	7, 71	0.46	1×10^{-4}	3×10^4	0.59	-0.003
Contact with another farm [‡]	1.95	1, 89	0.17	0.02	0.02	0.17	0.01
Presence of a water body [‡]	3.92	1, 81	0.05	0.04 (0 – 0.08)	0.02	0.05*	0.03
Grazing cattle away from the main herd [‡]	7.50	1, 87	0.01	0.04 (0.01 – 0.08)	0.02	0.01**	0.07
Use of the vaccine	1.00	1, 85	0.32	-0.02	0.02	0.32	<0.001
Anthelmintic frequency cows	0.16	2, 89	0.85	-	-	-	-
Anthelmintic frequency calves [‡]	2.38	2, 89	0.10	-	-	-	-

Significance: * $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

[‡]Denotes taken into multivariable regression

Table 4.6 Significant associations between exposure variables and pooled milk *Dictyocaulus viviparus* antibody titres using a stepwise multivariable linear regression modelling approach ($R^2=0.14$, $n=77$)

Exposure variable	Reference	B (95% CI)	Standard error	P value
Presence of beef animals	Absence	0.02 (-0.01 – 0.06)	0.02	0.18
Anthelmintic frequency in calves (/year)	None	Ref	-	-
	Once or twice	0.03 (-0.01 – 0.06)	0.02	0.16
	More than twice	-0.02 (-0.07 – 0.03)	0.03	0.39
Presence of water body	Absence	0.04 (0.00 – 0.08)	0.02	0.06
Grazing away from main herd	Not grazed away	0.04 (0.00 – 0.07)	0.02	0.04*

Significance: * $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

4.4 Discussion

The purpose of this study was to identify tangible farm management factors that could be targeted to control the risk of lungworm disease within herds. This nationwide cross – sectional survey of the UK’s grazing dairy herds has demonstrated that the presence of a water body and grazing cattle away from the main herd are both associated with a significant increase in lungworm antibody titres.

The significance of the presence of a water body was previously confirmed in a large scale study of 22,427 bulk tank milk samples from Germany (Schunn et al., 2013). Similar studies have found the presence of a water body increased antibody levels to *Fasciola hepatica* in bulk tank samples (Kuerpick et al., 2013) and that farms where either the heifers or dry cows grazed wet pasture had higher bulk tank antibody levels (Takeuchi-Storm et al., 2017). However, fewer studies have investigated the associations between the presence of a water body and increased antibody levels to *D.viviparus*. The *D.viviparus* lifecycle does not involve an amphibious intermediate host unlike for example, *Galba (Lymnea) truncatula* for *Fasciola hepatica* and so the role of rivers and ponds in lungworm epidemiology is less obvious. However, the mortality rates of trichostrongylid gastrointestinal nematodes in soil and water was 4.9 - 18.5 times lower than in faeces (Rose et al., 2015) and so it is conceivable that lungworm larvae may similarly survive for longer periods of time in boggy pasture than in dry ground. Lungworm larva, particularly the L1 and L2 stages, have been shown to be highly sensitive to desiccation (Daubney, 1920). Therefore, a strip of herbage adjacent to waterbodies, containing lush grass attractive to cattle, may provide high levels of infection to cattle in summer. During winter, the semi water – logged strips may provide an opportunity for larva to survive. Waterbody-adjacent pastures, where cattle have access to streams or waterbodies, may therefore provide an important complementary over-winter

survival mechanism for lungworm. Saatkamp et al (1994) proposed that over-winter survival of the parasite in the Netherlands depends mainly on carrier hosts. However, Dutch pastures are normally well drained and cattle rarely drink from streams. Certainly being able to survive over-winter on pasture would offer an evolutionary advantage to *D.viviparus*, ensuring that larva are consumed at turnout, thereby prolonging the window of opportunity for the transmission of the parasite and increasing the number of worm generations over a grazing season. Further work into the potential benefit in cornering off water bodies is needed, as this would be a useful, practical step in reducing the risk from several parasitic diseases.

Grazing a portion of the herd away from the main herd was also associated with a significantly increased antibody titre. This is the first study to find that grazing cattle away from the main milking herd, and subsequently being returned to the farm, as a significant risk factor for lungworm antibody titre. Bloemhoff *et al.* (2015) found that contract rearing of young stock was not significant in their regression modelling. In calf and prepubescent heifers were the age group most likely to graze away from the main herd in the present study. Adult carrier cows are thought to be vital in the year to year survival of lungworm larvae on a farm (Eysker et al., 1994b). A study looking at patent infections in adult cattle found that the spring emergence of faecal patency in cows occurred before they had been on pasture for the prepatent period of three weeks, suggesting that patency was due to maturation of inhibited larvae rather than overwintering of larvae (Eysker et al., 1994b). Yearling cattle which grazed without older cattle have been shown to be seropositive significantly less often than yearling cattle in mixed herds (Schnieder et al., 1993). Replacement heifers, which have not had this access to carrier animals, are likely to be naïve when they enter the milking herd, and this is one explanation for an apparent causative link between lungworm disease and grazing cattle away from the main herd. In this case, it would be the previously naïve heifers,

entering an infected herd, who would be responsible for the higher titres. However, another explanation may be heifers picking up larva while grazing on a different farm and shedding L1 in an adult herd with suboptimal immunity levels. Further work is needed to investigate the infection dynamics in heifers entering the adult herd.

This study provides some practical implications for control of the disease on farms. If heifers graze away from the farm and lungworm is present within the main herd, heifers should be vaccinated prior to joining the adult herd or monitored closely and selectively treated if they display suggestive clinical signs of the disease (particularly milk production losses or coughing). However, if lungworm is absent from the adult herd, heifers should be treated with anthelmintics prior to joining the adult herd. On farms where cattle graze next to a water body or have access to streams or boggy pasture, it is vital to test for the presence of lungworm, to be vigilant and to have a lungworm management protocol in place.

This study further showed that the awareness of the current lungworm status on modern UK dairy farms is poor, as reflected in the low sensitivity (45.9%) and specificity (40.0%) of a farmer's impression on whether lungworm was circulating on the farm. These findings were echoed in a recent DEFRA announcement declaring that "awareness of lungworm as a major cause of respiratory disease in grazing cattle may not be as high as perhaps it should be" (Department for Environment Food and Rural Affairs et al., 2014). The specificity of farmers reporting that animals had been coughing at pasture within the preceding 12 months was reasonably high at 73.2%. However, the low sensitivity of 23.5% suggests that herds containing cattle with potential clinical signs should undergo regular diagnostic screening. There is a great need for the application of improved herd-level presence absence tests that can routinely be employed to test a herd's lungworm status. It is hoped that this will result

in a greater importance being given to the disease in herd health plans and planned disease control strategies. The use of the lungworm vaccine should be a useful first step in enhancing a herd's immunity levels. Despite this, 62% of farmers did not use the vaccine. Further work should be targeted towards enhancing vaccine uptake. Several factors were listed as barriers to vaccination use. Some of these, particularly those listing logistical reasons with calving patterns and length of grazing season are difficult to overcome. However, other factors such as an overreliance on anthelmintic coverage or having never discussed the vaccine with their vet reflect that there is scope for veterinarians to improve communication surrounding lungworm, and lungworm awareness. Additionally anthelmintics were frequently used in adult cattle. The reasons driving this increased reliance on anthelmintics desperately need investigating, as does an accurate assessment on the impact that this has on herd immunity.

The use of directed acyclic graphs to inform statistical modelling techniques offers a valuable method in capturing the complexity of biological systems. Specifically, it offers a technique to apply logical reasoning, expert or commonplace opinion or prior assumptions in a fashion independent from statistical outcome. The traditional stepwise regression modelling presented here assumes that for example, the presence of beef animals, water body and grazing away from the main herd are all equal confounders on the causal pathway between anthelmintic frequency and lungworm antibody titre. These variables may themselves be confounded and this modelling technique does not allow for heterogeneity of confounders to the exposure of interest (Westreich and Greenland, 2013). Furthermore, inclusion of the variables into the multivariable model was based on the statistical outcome from univariable modelling, a technique itself which is influenced by statistical factors such as the data distribution and sample size. A limitation of the DAG approach would be the heavy reliance upon prior knowledge, which is influenced by previously published studies. There is a risk

that prior knowledge is influenced by variables that have gathered the largest amount of research interest rather than those with the largest biological significance. However, the transparency offered by the DAG approach should at least offer some biological reasoning behind model selection. Interestingly, the two approaches used here both identified that grazing away from the main herd was a significant risk factor whereas presence of a water body was significant according to the DAG approach and showed borderline significance according to multivariable analysis.

There are several limitations to this study including the relatively low sample size of 92 farmers which decreased the power of the study. Additionally, TSDG farmers tend to be high producing dairy farms and so the use of the TSDG community may have preselected farmers with a unique set of risk factors, which is unrepresentative of the larger dairy community. It is also possible that the use of TSDG for recruitment could have led some participants to feel under pressure to answer questions according to best practice policy. Furthermore, study participants were aware that the questionnaire was looking at risk factors for lungworm and so may have been biased in both who completed the questionnaire and the answers that were provided.

The fact that more variables, such as grazing length, anthelmintic protocols or vaccination use were not found to be significant in this study was perhaps surprising and may point to the low power of the study. However, several European risk factor studies have similarly found that only a few farm management risk factors were significant indicators of seroconversion (see for example Bloemhoff *et al.*, 2015; Frey *et al.*, 2018). The rapid population growth and decline of *D. viviparus* larvae on pasture (Daubney, 1920; Eysker *et al.*, 1994a; Jørgensen, 1980a) suggest that it should be easy to remove the parasite from a farm.

However, studies aimed at the eradication of larvae from pasture have not been successful (Eysker et al., 1997; Höglund, 2006). The low adjusted R^2 value within the multivariable model in the current study ($R^2 = 0.14$) suggests that unmeasured variables, such as climatic rather than farm – management variables, could play a large role in the introduction and maintenance of the disease on farm.

4.5 Conclusion

This is the first study in the field of human or veterinary parasitology to use a directed acyclic graph (DAG) approach in risk factor analysis. Grazing cattle at a distance from the main milking herd and the presence of a water body significantly increased the risk of lungworm disease according to pooled milk antibody titres. The low adjusted R^2 value in this study suggests that unmeasured variables, such as meteorological conditions, could play a large role in the risk of lungworm disease in dairy herds. Additionally, there were high levels of uncertainty amongst farmers on whether the parasite was circulating in their herd, suggesting that lungworm education should play a more active role in herd health planning and disease awareness.

4.6 Acknowledgements

The National Statistics Postcode Lookup (NSPL) for the United Kingdom relates current postcodes to a range of current statutory administrative, electoral, health and other statistical geographies via 'best-fit' allocation from the 2011 Census output areas. It supports the production of area based statistics from postcode data. The NSPL is produced by ONS Geography, which provides geographic support to the Office for National Statistics (ONS) and geographic services used by other organisations. Contains OS data © Crown copyright and database right [2018]

Chapter 5: LUNGWORM – FL: A climate based model of the free – living stages of *Dictyocaulus viviparus*

5.1 Introduction

Mathematical models provide a useful contribution to our understanding of parasite epidemiology (Verschave et al., 2016). They can be used to explore complicated trade-offs in biological systems, answer biological and epidemiological questions about the ecosystem in hand and predict short- and long-term trends in disease incidence rates and spatial trends.

There are several reasons why the modelling of *Dictyocaulus viviparus* epidemiology is much needed and is likely to harvest important new insights. When conditions are right for optimal disease transmission, clinical outbreaks can be explosive, unexpected and expensive with morbidity ranging from 6-100% (David, 1997). It is currently unknown which factors explain the annual differences in disease intensity but, in analogy with the gastrointestinal nematodes, the development, survival and migratory rates of the free-living stages are likely to be heavily influenced by local meteorological conditions on pasture (Jørgensen, 1980a; Rose, 1956). Previous studies have used multivariable (statistical) models to understand the effect of climatic variables on the presence of lungworm antibodies within bulk tank milk samples (Schunn et al., 2013), faecal larval output (Jiménez et al., 2007) and the numbers of infective third stage larvae on pasture (Somers and Grainger, 1988). However, only mathematical models are able to capture the complicated interactions between

meteorological conditions that can influence the development, survival and migration of parasites. Moreover, they facilitate an exploration of the build-up of larvae at pasture over several generations, enabling us to ask fundamental questions about lungworm epidemiology. Mathematical models present the user with the opportunity to understand how the transmission rates on pasture may alter under different climate conditions such as comparing different geographical locations or understanding the risk from potential climate change scenarios. Such models have previously been developed for the transmission of *Haemonchus contortus*, *Teladorsagia circumcincta* and *Ostertagia ostertagi* on pasture (Rose et al., 2015). Furthermore, the extent to which meteorological changes can explain the recent increase in cases of lungworm in northern England and Scotland (Veterinary Services, 2010) and the changes observed in the spatiotemporal distribution of cases in the Veterinary Investigation Diagnosis Analysis (VIDA) database (Chapter 2) can be explored using a modelling approach.

Furthermore, farm management practices such as the blanket application of anthelmintics, can prove costly and may interfere with the immune status of the herd (Ploeger et al., 2000). There is a need for an integrated management tool for lungworm, such as the NADIS parasite forecast (NADIS, 2018) which can predict the risk on individual farms and can be used to accurately target treatments to cover periods of peak larval abundance at pasture. An early warning surveillance system for lungworm would aid risk management decisions by veterinarians and farmers to maximise the economic benefit from application of anthelmintics. Alternatively, farms that are looking to reduce their reliance on pharmaceutical control measures may be able to use such a model to base decisions on when to rotate or rest pastures.

Although cattle mount protective immunity against lungworm during infection, such immunity is short-lived. Immunity to incoming larvae wanes after a few months whereas immunity to adult worms disappears over a time frame of two years (Michel and Mackenzie, 1965). Therefore, prevention of clinical disease depends not on the complete elimination of the parasite from the farm but, instead, on the maintenance of a low level of transmission between cattle sufficient to sustain immunity. Ultimately, this fine balance could be investigated in a safe manner using a combined mathematical model of the free-living and host stages.

The aim of this chapter is to develop a robust mathematical model of the free-living stages of *D.viviparus* that can predict the risk of lungworm disease based on local climatic conditions. Although a model has previously been developed of the parasitic stages of the disease (Ploeger and Eysker, 2000), none such model exists for the free-living stages. Moreover, the model will capture the critical role that the *Pilobolus* spp. fungus has on the epidemiology of lungworm. The LUNGWORM-FL model to be developed will be an adaptation of the previous GLOWORM-FL model. This chapter will have three major sections: model parameterisation and development, validation and application. Parameters will be estimated from the published literature and results derived from specifically designed laboratory studies. The developed model will subsequently be used to explore the recent change in disease prevalence across Great Britain and predict changes under future climate change scenarios. This model should drive accurate and informed decision making in the face of a challenging and evolving epidemiological picture. It is hoped that this will have direct, practical use for both veterinarians and dairy farmers in predicting heightened risk periods and developing targeted treatment plans. Specific objectives are to measure the degree to which the timing, intensity between years, and spatial distribution of cases can be explained

by climatic variables. This can then be used to assess the likely changes in lungworm epidemiology under climate change scenarios.

5.2 Parameterisation and model development

5.2.1 Methods

LUNGWORM-FL is a framework of the free-living stages of the lifecycle of D.viviparus which has been adapted from the GLOWORM-FL model (Rose et al., 2015). The model was parameterised by collecting data from published studies that reported either the development, mortality or migratory rates of the various larval stages. Laboratory studies were designed when appropriate literature was unavailable. Assumptions of the model are shown in Table 5.1.

5.2.1.1 LUNGWORM-FL model framework

First stage larvae (L1) are shed in the faeces and develop through to second (L2) and third stage (L3f) larvae as a function of mean daily soil temperatures (°C) (Figure 5-1). The model assumes that L1 are deposited in the faeces and ignores the occasional account of development through to the L2 stage in the egg during early laboratory studies (Daubney, 1920). The model allows for newly deposited L1 (L1new) to enter the pre-existing pool.

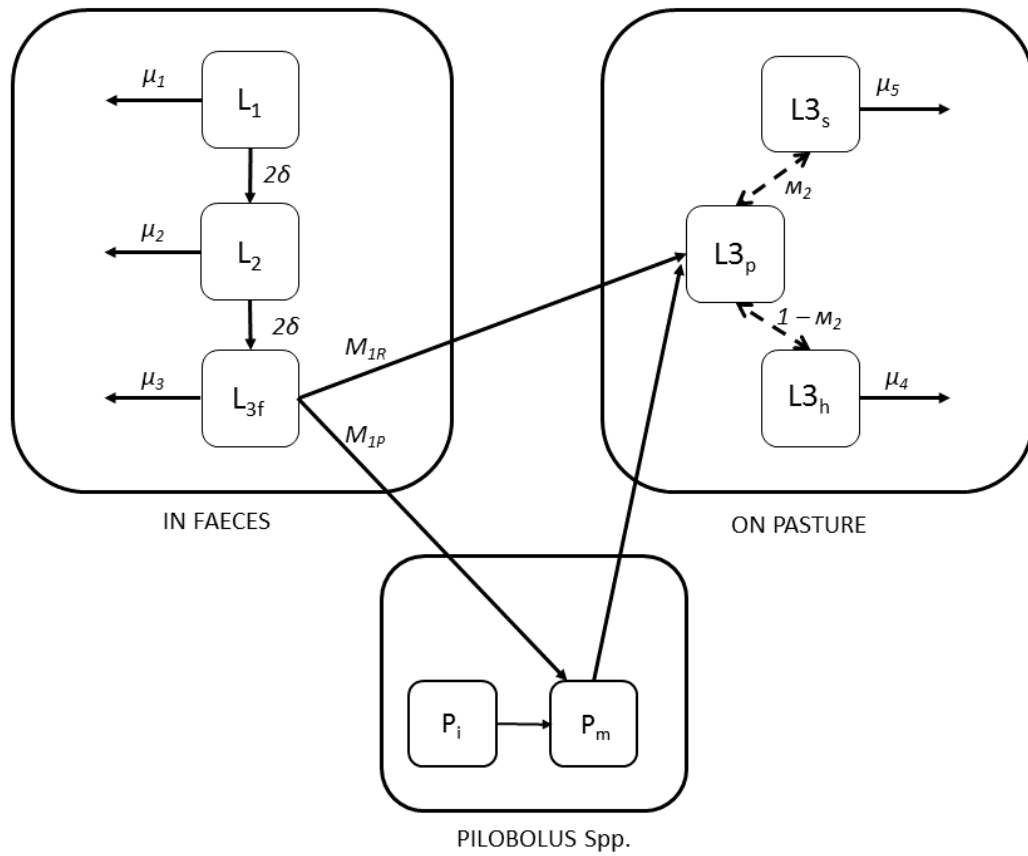


Figure 5-1 Conceptual diagram of LUNGWORM-FL model framework showing stages in faeces, with *Pilobolus* spp. and on pasture. See Table 5.1 and Table 5.2 for definitions of parameters and state variables.

Table 5.1 Assumptions of the LUNGWORM – FL model

Assumptions of the LUNGWORM – FL model
L1 are deposited in the faeces
Development and mortality rates are dependent upon the mean daily soil temperature
Migration is dependent upon the mean daily soil temperature and total daily rainfall
There is sufficient moisture within cattle faeces for development to occur and increased rainfall will not affect development or mortality rates
<i>Pilobolus</i> spp. are always present in the cattle faeces
There is no upper limit to the number of L3 which can be translated by the <i>Pilobolus</i> spp. fungus in any 24- hour period
<i>Pilobolus</i> spp. which have developed at different temperatures have an equal ability to disperse larvae onto pasture
If <i>Pilobolus</i> spp. have broken through the protective crust, which may form on the surface of cattle faeces under conditions of low rainfall, then there is sufficient breakdown of the crust for L3 larvae to migrate up the sporangia
There is no independent migration of L3 larvae except under rain splash dispersal
Cattle will graze herbage of any height
Animals are set stocked at pasture
During climate change predictions, there will be no evolutionary adaptations from <i>D.viviparus</i> larvae
During climate change predictions, there will be no evolutionary adaptations from <i>Pilobolus</i> spp. fungus
Climate change will progress according to the Intercomparison Project Phase 5 (CMIP5) Representative Concentration Pathway 8.5 (RCP 8.5) predictions

It has previously been assumed that moisture is not a limiting factor for gastrointestinal nematode development within cattle faeces at low faecal moisture contents (Rose, 1961) because a protective crust quickly forms on the surface of the pat and the decrease in faecal moisture content within cattle faeces is gradual (Mauleon and Gruner, 1984). Therefore, development and mortality rates of *D.viviparus* were assumed to be dependent upon temperature conditions only (Table 5.1). Development through the preinfective stages is modelled by the following equations:

$$\frac{dL1}{dt} = -(\mu_1 + 2\delta)L1 + L1_{new} \quad \text{Equation 5}$$

$$\frac{dL2}{dt} = -(\mu_2 + 2\delta)L2 + 2\delta L1 \quad \text{Equation 6}$$

$$\frac{dL3_f}{dt} = -(\mu_3 + m_{1P} + m_{1R})L3_f + 2\delta L2 \quad \text{Equation 7}$$

The total number of L3 on pasture ($L3_p$) were assumed to either reside in the herbage ($L3_h$) or on the soil layer ($L3_s$). Two migration rates were included in the model. The horizontal migration rate incorporated the translation rate of *D.viviparus* L3 larvae from the faeces to the herbage. *D.viviparus* larvae display a poor ability to migrate independently from faecal pats to herbage unless rainfall is heavy and larvae are washed out from faeces (Rose, 1956) or the synergistic fungus *Pilobolus* spp. is present (Somers and Grainger, 1988). Therefore, the horizontal migration rate incorporates the migration due to *Pilobolus* spp. (m_{1P}) plus the contribution due to rain splash dispersal (m_{1R}) and was modelled as a function of mean daily temperature and total daily precipitation. The second, vertical, migration rate (m_2) allowed for movement of L3 from herbage to soil according to mean daily temperature. The total number of L3 on pasture was modelled by the following equation:

$$\frac{dL3_p}{dt} = -\mu_4 \left((1 - m_2)L3_p \right) - \mu_5 (m_2 L3_p) + L3_f (m_{1P} + m_{1R}) \quad \text{Equation 8}$$

State variables and parameter definitions are listed in Table 5.2. The model was implemented in R (RCoreTeam, 2017) using the *gloworm* package.

Table 5.2 State variable and parameter definitions for the LUNGWORM-FL model. Full parameter estimates are given in Table 5.3.

State variable / parameter	Definition	Units	Parameter depends upon
L_1	First stage (L1) larvae	-	-
L_2	Second stage (L2) larvae		
L_{3f}	Third stage infective larvae (L3) in faeces	-	-
L_{3p}	Total L3 on pasture (soil and herbage combined)	-	-
L_{3s}	L3 in soil	-	-
L_{3h}	L3 in herbage	-	-
P_i	Immature <i>Pilobolus</i> spp. sporangia	-	-
P_m	Mature <i>Pilobolus</i> spp. sporangia	-	-
δ	Development rate from L1 to L3	Instantaneous daily rate	Mean daily temperature (°C)
μ_1	L1 mortality rate	Instantaneous daily rate	Mean daily temperature (°C)
μ_2	L2 mortality rate	Instantaneous daily rate	Mean daily temperature (°C)
μ_3	L3 mortality rate in faeces	Instantaneous daily rate	Mean daily temperature (°C)
μ_4	L3 mortality rate in herbage	Instantaneous daily rate	Mean daily temperature (°C); Ultraviolet (UV) light
μ_5	L3 mortality rate on soil	Instantaneous daily rate	Mean daily temperature (°C)
m_{1P}	Horizontal migration (translation) of L3 onto pasture due to <i>Pilobolus</i> spp.	Instantaneous daily rate	Mean daily temperature (°C)
m_{1R}	Horizontal migration (translation) of L3 onto pasture due to rain splash dispersal.	Instantaneous daily rate	Total daily rainfall (mm)
m_2	Proportion of total pasture L3 on soil	Proportion	-

5.2.1.2 Temperature dependent development and mortality rates in faeces and herbage

Temperature-dependent instantaneous daily rates of development from L1 to L3 (δ) and mortality rates of L1 and L2 (μ_1 , μ_2) and L3 in faeces (μ_3) were estimated from published experiments which reported development or survival rates at a constant temperature. For studies that reported the time to 50% development (D50) or mortality (M50), the instantaneous daily rates were estimated for each constant temperature as:

$$\text{Instantaneous daily rates} = -\frac{\ln(0.5)}{D50} \quad \text{or} \quad -\frac{\ln(0.5)}{M50} \quad \text{Equation 9}$$

If studies reported the proportion remaining at a single sampling interval, rates were calculated as:

$$\text{Instantaneous daily rates} = -\frac{\ln(\text{proportion remaining})}{\text{days}} \quad \text{Equation 10}$$

Alternatively, if studies reported the proportion developing or remaining (y) over several days, rates were calculated using the following equation described by Azam et al. (2012):

$$x = \log_{10}\left(\frac{y}{1-y}\right) \quad \text{Equation 11}$$

The instantaneous daily rates were calculated by linear regression of x against day and then identifying the intercept on the horizontal axis (day at which 50% developed or remaining).

A linear model was fitted to the instantaneous daily development rate at a range of temperatures to yield a regression equation, which could estimate daily rates given observed temperature data. Polynomial models were fitted to the log-transformed mortality rates and limited between 0 and 1 where necessary.

Rose (1956) reports mortality rates of L3 in both wet and dry faeces in incubator studies. It was assumed that laboratory studies with smaller quantities of faeces have fewer opportunities to create a defensive surface crust and so incompletely replicated field conditions. Therefore, the data from both wet and dry faeces were combined to parameterise the mortality of L3 in faeces (μ_3).

No data was available for the mortality rates of L1/L2 in faeces (μ_1 , μ_2) at temperatures between 0°C and 37°C. Therefore, the difference in mortality rates between L1/L2 (μ_1 , μ_2) and L3 in faeces (μ_3) at -7°C was calculated to create a conversion factor to infer the mortality rates of L1/L2 at 10°C, 20°C and 30°C from the mortality of L3 in faeces. Mortality rates at -7°C were chosen because this was the only temperature where mortality of both L1/L2 and L3 had been assessed in a published laboratory study.

No data was available for 100% mortality rates of L3 in faeces (μ_3). Larvae have been shown to be transmitted at 31.3°C (Jimenez et al., 2008) and survive temperatures as low as -7°C (Rose, 1956). However, *D.viviparus* infective larvae only survived a few days at 35°C in slurry (Olsen and Nansen, 1987). Therefore, maximum instantaneous daily mortality rates of 1 were applied at 35°C and -15°C to allow for a small survival rate at 31.3°C and -7°C.

No data was available on the mortality rates on herbage (μ_4). It was assumed that mortality on herbage would be higher than on soil because ultraviolet light exposure increases the mortality of infective trichostrongyloid L3 larvae by 2.27 times that of sheltered controls (van Dijk et al., 2009). Similarly, high temperatures and lack of oxygen mean that *D.viviparus* larvae are rapidly deactivated in slurry (Olsen and Nansen, 1987). Therefore, in a similar fashion as for GLOWORM-FL (Rose et al., 2015), the mortality of L3 on herbage (μ_4) was assumed to be as high as in faeces (μ_3).

5.2.1.3 Temperature dependent mortality rates in soil

For GLOWORM-FL, the mortality rates of gastrointestinal L3 in soil were estimated from studies reporting the mortality of L3 in water (Rose et al., 2015). As this data was unavailable for *D.viviparus*, laboratory studies were performed. First stage larvae from experimentally infected calves were incubated through to the L3 stage in fresh water at the University of Hannover, Germany, and dispatched via overnight courier to the University of Liverpool. Ice packs were used during transit to keep the faeces chilled. A pure culture of approximately 19,500 third-stage larvae in water was received (approximately 170 larvae/ml). Three replica Petri dishes, each containing approximately 700 L₃ larvae were placed in incubators at 5, 10, 15, 20, 25 and 30°C and the lights switched off. Samples were examined at the start of the experiment and then every other day and distilled water was added as required to prevent desiccation. Each week 50 larvae from each Petri dish were examined in a 1ml nematode slide under 100x magnification and the proportion of living larvae counted. Larvae were assumed to be dead if either in a characteristically stretched out position or if damage to the body wall was visible (Figure 5-2). Any remaining larvae were stored at 5°C in aliquots containing 1ml distilled water. The experiment was then repeated at freezing temperatures of 0°C, -2°C and -4°C. On days 1, 3, 5 and 22, two aliquots were removed from the incubators,

placed at 5°C for two hours to acclimatise to the increased temperature, and counted as previously described.

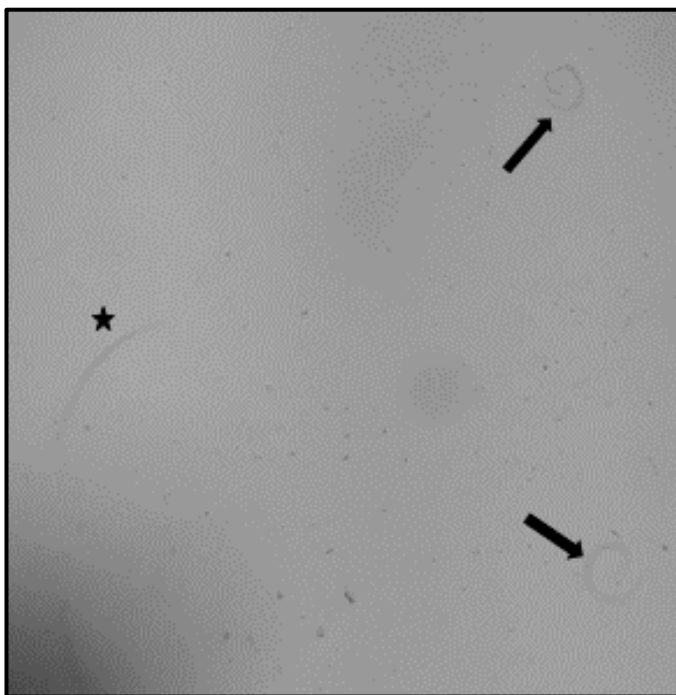


Figure 5-2: Microscopic picture of Dictyocaulus viviparus larvae showing living L3 larvae (arrows) and dead L3 larvae (stars) (magnification x100) [Photograph: C. McCarthy]

To examine the effect of desiccation on L3 survival, 1ml of approximately 70 larvae/ml solution was placed in Petri dishes in incubators at 15, 20 and 25°C. The Petri dish lids were removed to allow the water to evaporate. At 1, 3 and 10 days post-incubation, two dishes were removed from each incubator. The addition of 2ml water to the dry Petri dishes, and continuous homogenization, facilitated larvae to be collected and aspirated onto a 1ml nematode counting slide. The proportion of living larvae was recorded as before.

The instantaneous daily mortality rates at each constant temperature were estimated by the method described by Azam et al. (2012). Mann-Whitney U tests were used to assess whether the instantaneous daily mortality rates were significantly different under wet and dry conditions at 15, 20 and 25°C. Data points were combined in the event of no significant difference. A polynomial model was fitted to the log-transformed mortality rate.

5.2.1.4 Temperature and moisture limitations on the migration of L3 from faeces to herbage and soil

5.2.1.4.1 *Horizontal migration due to Pilobolus spp.*

The translation of L3 onto pasture is intrinsically linked to the synergistic fungus, *Pilobolus* spp. (Doncaster, 1981; Grønvold and Jørgensen, 1987b; Jørgensen et al., 1982; Robinson, 1962). Laboratory studies were performed to calculate the development and sporulation rate of *Pilobolus* spp. under a range of constant temperature and rainfall conditions. Fresh faeces from dairy cattle naturally infected with *D.viviparus* was collected from a farm in Leicestershire, UK. A sensitive flotation procedure was used to confirm the presence of the first-stage larvae in the faeces (Great Britain. Ministry of Agriculture, 1986).

Three Petri dishes each containing 10g faecal cultures were placed in incubators at each of 10, 15, 20, 25 and 30°C. Incubators were set to have a 12-hour fluctuating light on, off cycle. Cultures were checked on a daily basis and sprayed with a fine horticultural sprayer from a height of approximately 30cm if deemed to be drying out. Petri dish lids were replaced with fresh lids at a set time every day. Pilot experiments showed that the number of sporangiophores per 10g faecal culture was very large, sometimes exceeding 6,000 sporangiophores but that these easily became attached to the Petri dish lid (Figure 5-3). It

was not possible to count all discharged sporangiophores because sporangia were often seen aligning horizontally and therefore discharging onto the wall of the Petri dish. Therefore, in order to gain a semi-quantifiable assessment of *Pilobolus* spp. sporulation rate at different temperatures, an opaque paper cover slip was produced which would fit over the petri dish lid with three cut – out windows in which to isolate three views into the Petri dish lid. The number of discharged sporangiophores in each window was then counted to calculate an average daily sporulation rate per 24 – hour period.

In order to mimic variations in rainfall, additional faecal cultures were placed in an incubator at 20°C with a 12-hour fluctuating light on, off cycle. Two cultures were watered with three pumps from a fine horticultural sprayer every other day and were deemed control samples. Two were watered every fourth day and a further two were watered every seventh day. Experiments were terminated when the sporangiophores stopped discharging sporangia for two consecutive days.

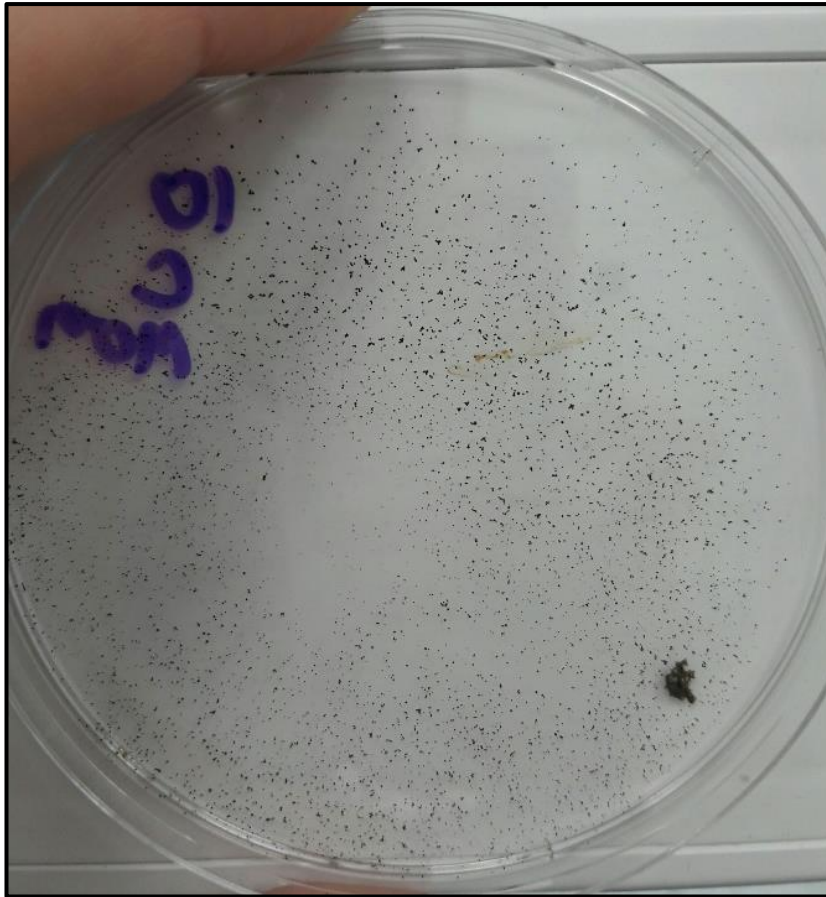


Figure 5-3: Photograph showing a typical daily dispersal rate of mature sporangia onto the lid of a Petri dish over a 24-hour period. Sporangiphores had grown on cattle faeces in incubators at 10°C. An abundance of sporangia are present on the lids with more visible on the petri dish walls. [Photograph: C. McCarthy]

The horizontal migration rate (translation from faeces to pasture) due to *Pilobolus* spp. (m_{1p}) was calculated as a function of the proportion of sporulations multiplied by the day at which 50% of the sporangiphores had discharged at each temperature. The proportion of sporulations was calculated by taking the total number of sporulations at each constant temperature compared to the temperature with the highest total sporulations. As the number of larvae that can occupy one sporangiphore is unknown, there was no upper limit applied to the number of larvae that could migrate in one day. Mann-Whitney U tests were used to assess whether the sporulation rates were significantly different under wet and dry conditions. Data points were combined in the event of no significant difference. A polynomial

model was fitted to the log – transformed migratory rate to calculate the instantaneous daily migration rate due to *Pilobolus* spp. translation (m_{1P}).

For many gastrointestinal parasites of sheep and cattle, larvae can develop within the faeces in the absence of a significant rainfall event, but will be unable to migrate onto the pasture until sufficient rainfall disintegrates the protective crust (Khadijah et al., 2013; O'Connor et al., 2008). This often leads to mass emergence of infective larvae onto pasture. However, for *D.viviparus* under low rainfall conditions, it was assumed that the action of *Pilobolus* spp. developing on the surface of the faeces would disrupt the protective crust sufficiently to allow L3 to migrate onto the fungus. Therefore, migration due to *Pilobolus* spp. was modelled as a function of temperature and rainfall only if water conditions significantly altered the sporulation rate of the fungus in the experiments.

5.2.1.4.2 *Horizontal migration due to rain splash dispersal*

In the absence of *Pilobolus* spp., Somers and Grainger (1988) placed 200g faeces containing 176 L1 larvae/g on pasture and recovered 39 L3 larvae/kg on the surrounding herbage after 7 days. The study used a herbage collection technique with a reported recovery rate of 60% (Jørgensen, 1975b). In order to calculate the predicted total number of L3 within the faeces, the LUNGWORM-FL model was run with migration due to *Pilobolus* spp. (m_{1P}) set to zero using meteorological data taken from the nearest weather station (Dublin airport at a distance of 7km from study location) for the date of the study (commencing 25th July 1984). Meteorological data was obtained from the E-OBS gridded dataset (Haylock et al., 2008). The migration rate in the absence of *Pilobolus* spp. was calculated using Equation 10 as:

$$\text{Instantaneous daily migration rate} = -\frac{\ln(1 - (L3 \text{ on herbage} / \text{total L3 in faeces}))}{7}$$

It is rare for larvae to move independently from faecal pats to the herbage except when rainfall is heavy (Rose, 1956). Therefore, the migration rate depicted during the Somers and Grainger (1988) study was assumed to be due to rain splash dispersal. Gastrointestinal nematodes have been shown to migrate from cow faeces following 1.6mm of simulated rainfall (Grønvold and Høgh-Schmidt, 1989) and from sheep faeces following 2mm of simulated rainfall (Rose et al., 2015). Therefore, an optimal total rainfall of 2mm per day was predicted to allow migration due to rain splash dispersal, as per Rose *et al.* (2015).

5.2.1.4.3 Vertical migration from herbage to soil

Very few studies consider the vertical migration rates of nematodes between the soil and herbage. Callinan and Westcott (1986) counted the vertical distribution of trichostrongylid larvae on herbage and soil which enabled Rose *et al.* (2015) to develop a vertical migration parameter for their model. *D.viviparus* larvae have very limited migratory ability, with only 7 – 15% of *D.viviparus* larvae recovered at a height above half an inch from the soil (Rose, 1956), compared to 29% of recovered trichostrongylid larvae present at 2cm above the soil layer at 20°C (Callinan and Westcott, 1986). This implies that at 20°C, between 20 – 50% fewer *D.viviparus* larvae will have migrated vertically than trichostrongylid larvae. Therefore the vertical migration equation used by Rose *et al.* (2015), modelled as a function of mean daily temperature, was multiplied by 0.2 to replicate the reduced migration.

5.2.1.5 Validation of parameters

For initial parameter validation, the number of larvae predicted to develop and survive within the faeces and migrate onto the pasture were assessed for correlation with published studies. Studies that a) monitored the development, survival or migration of any larval stage; b) were conducted under field conditions; and c) had not previously been used in the parameter estimates, were used to validate the parameters. Note that data for validation of parameters was independent from data used to develop parameter estimates even if discussed in the same paper. Rose (1956) placed faeces containing 600 L1 larvae on herbage in boxes placed outdoors and counted the total number of larvae surviving in the faeces on a weekly basis. In a separate experiment, faeces containing 3,000 L1 larvae was spread onto herbage and the number of L3 that had dispersed onto one quarter of the herbage was counted on a weekly basis. This experiment used a technique with a 60% success rate at isolating larvae from herbage (Jørgensen, 1975b) and so study results were modified as appropriate. Each experiment was repeated with four replicates in July and October (assumed 1st July and 1st October 1954 respectively). The mean and 95% confidence intervals for each week's data from the four replicates was calculated. LUNGWORM-FL was used to predict the number of larvae developing and surviving using the meteorological data which was collected from the E-OBS dataset (Haylock et al., 2008). Latitude and longitude coordinates were taken from the study location at the Central Veterinary Laboratories, Weybridge (lat/lon: 51.355°N, -0.496°E). As these were field experiments using natural cattle dung, it was assumed that *Pilobolus* spp. would be present and would contribute towards predicted migration. Spearman's correlation tests were used to assess the model fit between modelled outcome and study results.

5.2.2 Results

5.2.2.1 Development and sporulation rate of *Pilobolus* spp.

The development of *Pilobolus* spp. sporangiophores begins with immature, yellow sub – sporangial swellings that develop black sporangium once mature (Figure 5-4). Dispersed sporangia attached firmly to the Petri dish lid or walls. It was observed that there was a difference in size of discharged sporangia under different temperature settings, with those developing at 10°C appearing particularly small. Whether this has an impact on the translation of *D.viviparus* larvae onto the pasture is unknown and so was not included as a term in LUNGWORM-FL.

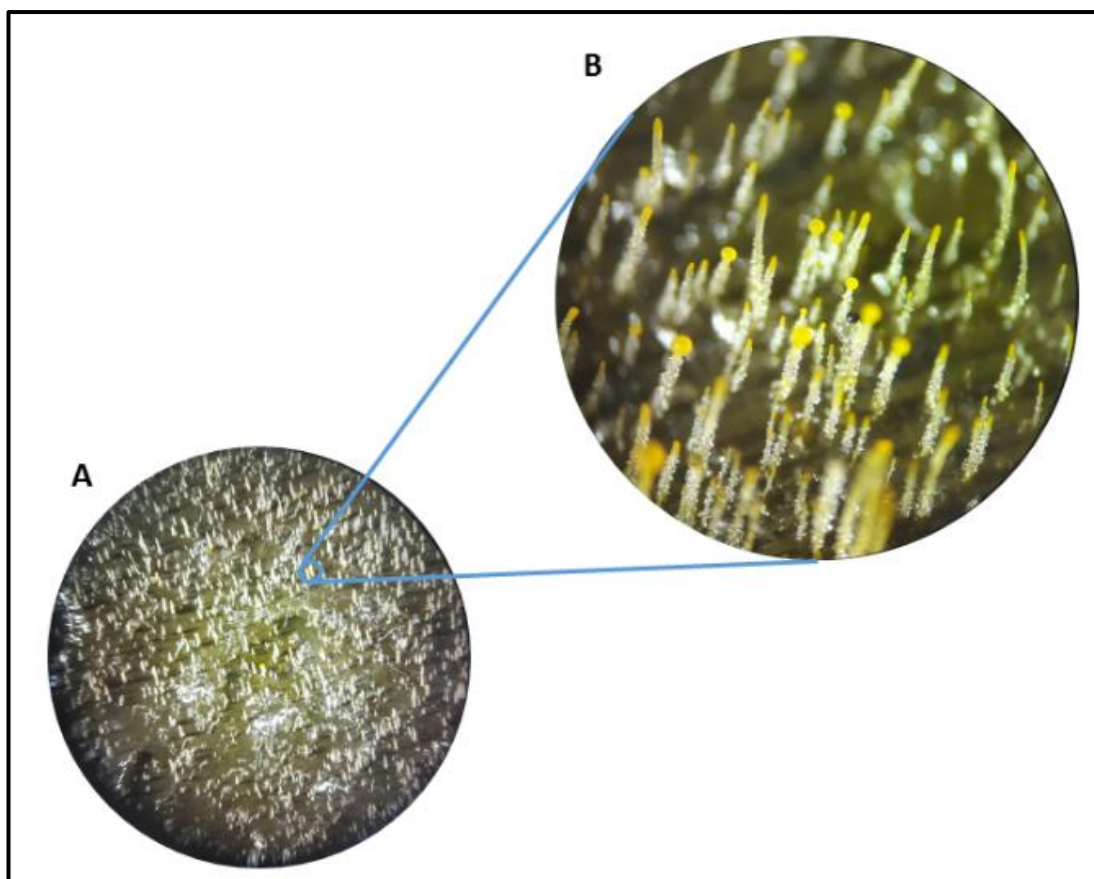


Figure 5-4: Growth of *Pilobolus* spp. on cattle faeces after two days in an incubator at 20°C. Microscopic photographs taken at (A) x8 magnification and (B) x32 magnification showing abundant growth of immature sporangiophores with yellow sub-sporangial swellings and occasional mature black sporangium. [Photograph: C. McCarthy]

The fewest sporulations occurred at 30°C with 120.3 sporangia / 10g faeces (95% confidence intervals 71.4 - 205.3) with the highest number occurring at 20°C with 9735.7 sporangia / 10g faeces (95% confidence intervals 9513.8 - 10052.5) (Figure 5-5). The time to 50% sporulation was shortest at both 30°C (2.3 days, 95% confidence intervals 2 - 3) and 25°C (2.3 days, 95% confidence intervals 2 - 3) and longest at 10°C (8 days, 95% CI 8 - 8). There was no significant difference in *Pilobolus* spp. sporulation rate between watering every second and fourth day ($U=332.5$, $p=0.93$), second and seventh day ($U=301.0$, $p=0.50$) or watering every fourth and seventh day ($U=319$, $p=0.73$). Therefore, horizontal migration due to *Pilobolus* spp. was modelled as a function of mean daily temperature and not rainfall.

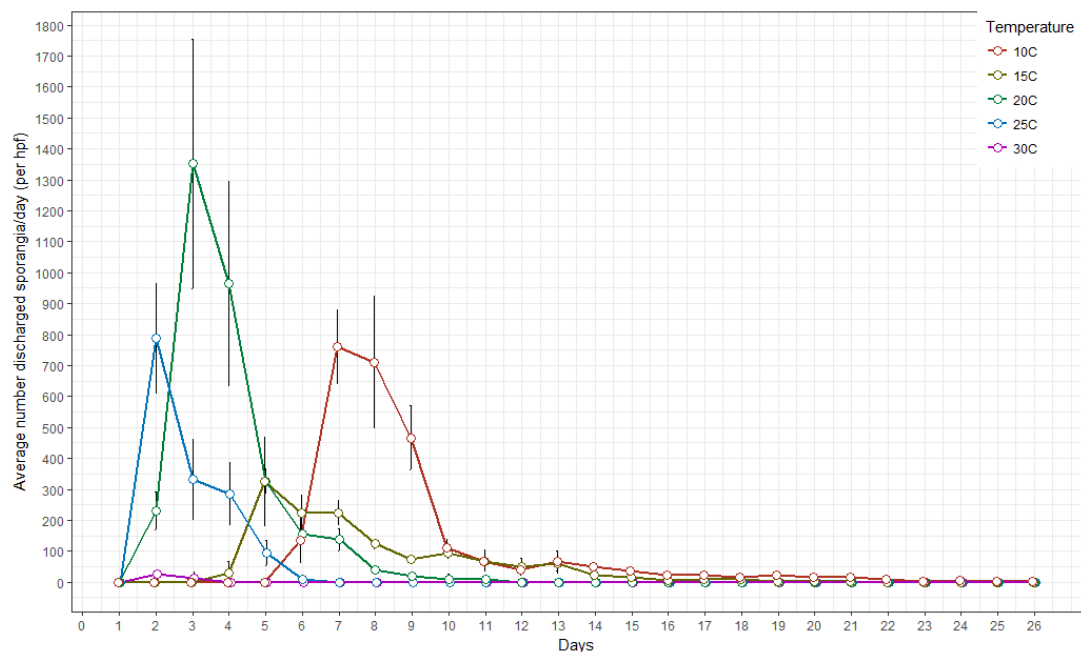


Figure 5-5: *Pilobolus* spp. sporulation rate (number discharged sporangia over 24 – hour period) when growing on cattle faeces at constant temperatures in an incubator. Vertical error bars show standard deviation.

5.2.2.2 Parameterisation of model

Parameter estimates and the corresponding modelled equations can be seen in Figure 5-6 and Table 5.3. There was no significant difference in L3 mortality under wet or dry conditions in soil (μ_5) at 15°C (U=5, p=1), 20°C (U=6, p=0.84), or at 25°C (U=2, p=0.32) and so data points for both wet and dry conditions were combined to deduce an overall mortality rate of L3 in soil (μ_5). Larval development rate (δ) was best fitted with a linear model with a minimum temperature for development of 1.39°C.

Table 5.3 Parameter estimates for the LUNGWORM-FL model showing the data source and model equations. See Table 5.2 for state and parameter definitions.

Parameter	Estimate ^b	Data source
δ	$-0.0154 + 0.0111T$ ($F_{1,5}=59.7$, $p<0.001$, $R^2_{adj}=0.91$)	Rose (1956), Jørgensen (1980)
μ_1	$\exp(-1.1725 - 0.0638T + 0.0026T^2)$ ($F_{2,5}=12.0$, $p=0.23$, $R^2_{adj}=0.22$)	Daubney (1920), Rose (1956)
μ_2	As μ_1	-
μ_3	$\exp(-2.018659 - 0.086970T + 0.004394T^2)$ ($F_{2,7}=3.6$, $p=0.09$, $R^2_{adj}=0.36$)	Rose, 1956; Olsen and Nansen, 1987; Jiménez <i>et al.</i> , 2007
μ_4	As μ_3	-
μ_5	$\exp(-3.3064 - 0.0863T + 0.0043T^2)$ ($F_{2,28}=16.8$, $p<0.001$, $R^2_{adj}=0.51$)	Current study
m_{1P}	$-0.3277 + 0.0489T - 0.0012T^2$ ($F_{2,12}=15.4$, $p<0.001$, $R^2_{adj}=0.67$)	Current study
m_{1R}	$\begin{cases} 0.0012, & P \geq 2 \\ 0, & P < 2 \end{cases}$	Somers and Grainger, (1988)
m_2	$0.2 (\exp(-5.4824 + 0.45392T - 0.01252T^2))$	Callinan and Westcott (1986)

T = mean daily temperature (°C); *P* = total daily precipitation (mm)

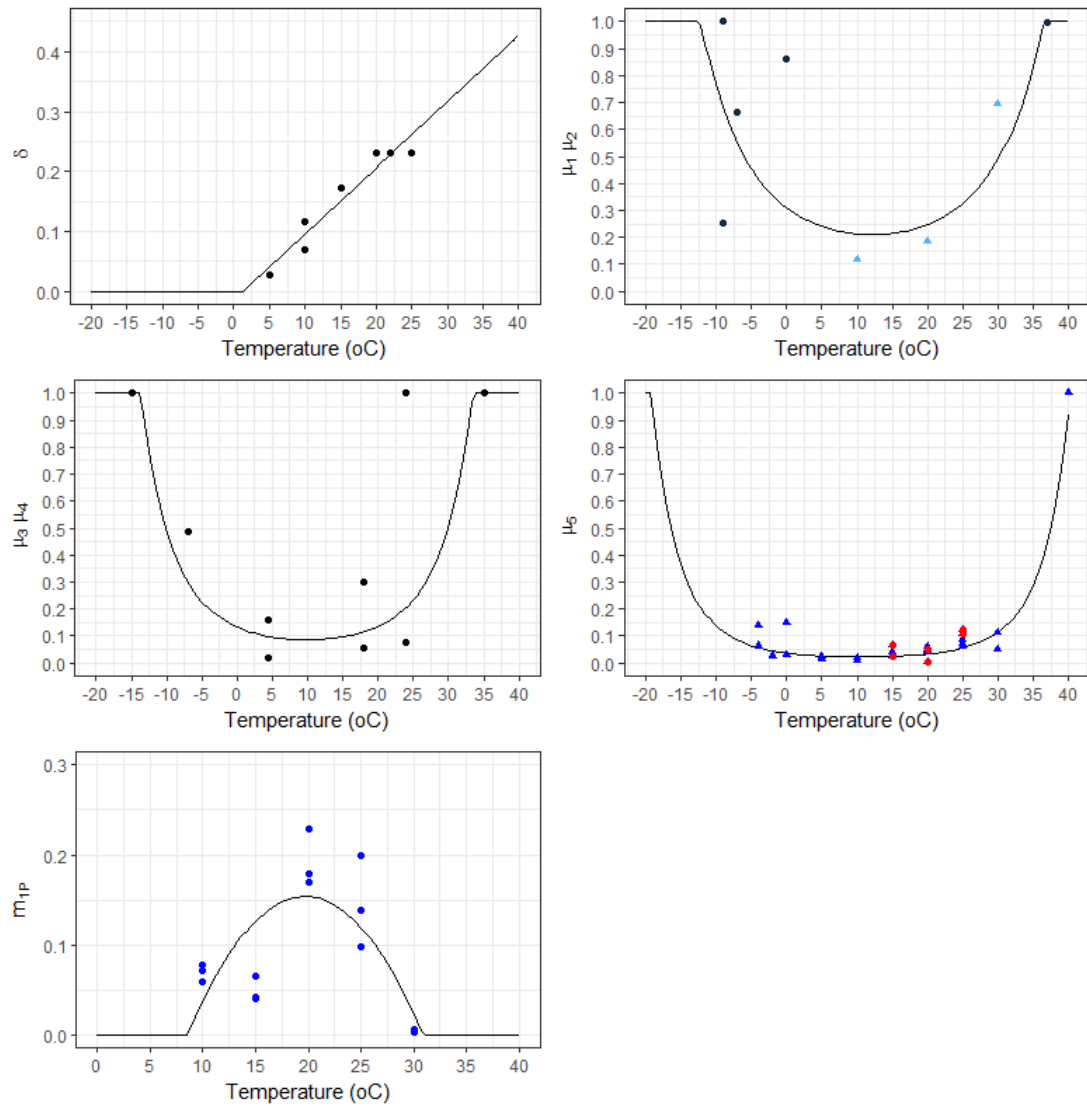


Figure 5-6: Temperature-dependent parameter estimates for the free-living stages in the lifecycle of *Dictyocaulus viviparus*. See Table 5.2 and Table 5.3 or an explanation of state variables and the modelled equations respectively. Data points obtained from published studies are displayed as black circles. Data was unavailable for the instantaneous daily mortality rates of L1 and L2 in the faeces (μ_1 , μ_2) for temperatures between 0-37°C and so mortality rates have been implied (blue triangles) based on the ratio of mortality rates between L1 (μ_3) and L3 in the faeces (μ_3) at -7°C. Data was obtained for the mortality of L3 in the soil (μ_5) based on laboratory experiments under dry (red circles) and wet (blue triangles) conditions. Horizontal migratory rates due to *Pilobolus* spp. sporulation (m_{1p}) are based on laboratory experiments designed to find the total number of sporulations and the time to 50% sporulation (days) at each constant temperature (see text).

5.2.2.3 Validation of model parameters

Validation of the lifecycle stages which occur within the faeces, namely development (δ) and mortality (μ_1, μ_2, μ_3) of the larval stages, can be compared to field studies (Figure 5-7). There was a strong correlation between the modelled output and number of larvae counted in the summer ($r_s=0.97, p<0.001$) and in the winter ($r_s=0.91, p<0.001$).

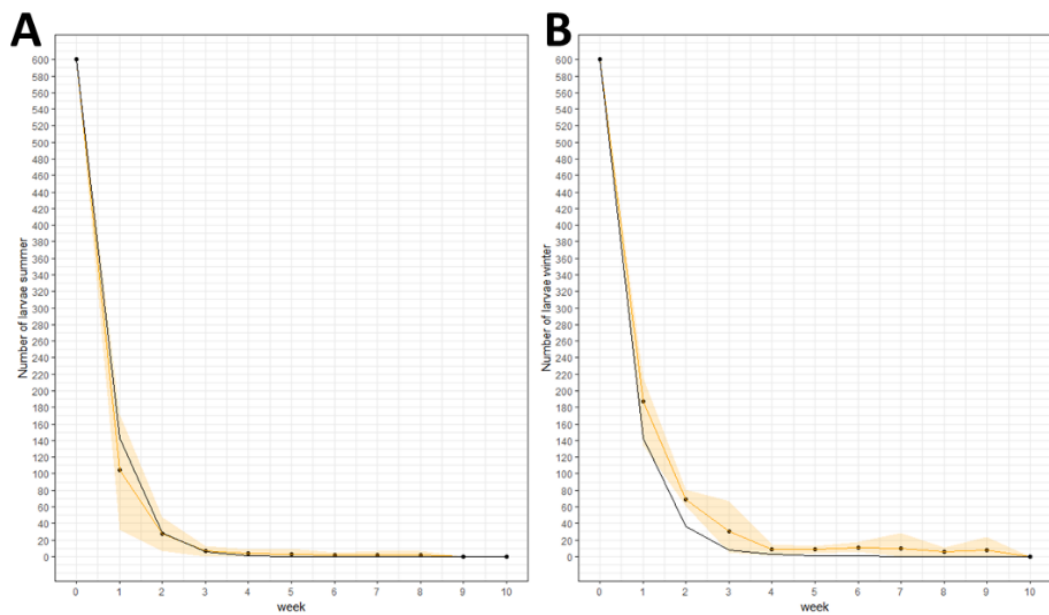


Figure 5-7: Mean (orange line) and 95% confidence intervals (orange shading) of numbers of larvae surviving in faeces placed outdoors at the Central Veterinary Laboratories, Weybridge on either A) the 1st July 1954 or B) the 1st October 1954 according to data collected by Rose (1956). Black line shows model predictions according to LUNGWORM-FL for the same period using meteorological data from the E-OBS gridded dataset.

Validation of the lifecycle stages which occurred outside of the faeces, including the horizontal migration due to *Pilobolus* spp. or rain splash dispersal (m_{1P} , m_{1R} respectively), vertical migration from herbage to the soil (m_2) and L3 mortality in the herbage and soil (μ_4 , μ_5 respectively) can be seen in Figure 5-8. There was a strong significant correlation in the summer ($r_s=0.96$, $p<0.001$) but not in the winter ($r_s=0.35$, $p=0.33$). The model predicted that more larvae would migrate onto the pasture in the summer and less would migrate in the winter than was found in the published studies.

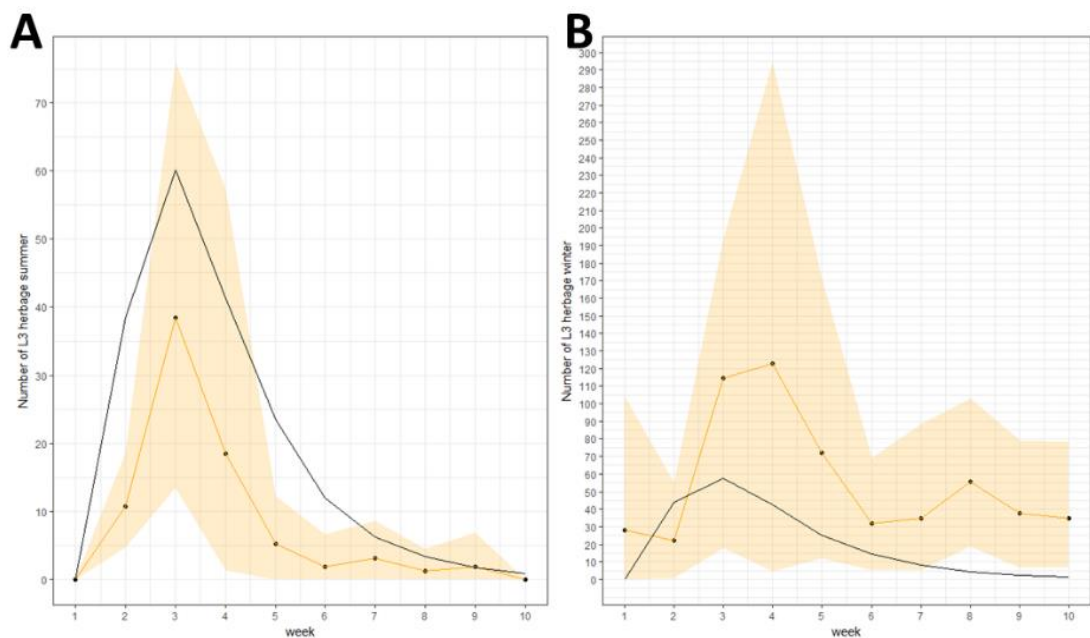


Figure 5-8: Mean (orange line) and 95% confidence intervals (orange shading) of numbers of larvae surviving on herbage placed outdoors at the Central Veterinary Laboratories, Weybridge on either A) the 1st July 1954 or B) the 1st October 1954 according to data collected by Rose (1956). Black line shows model predictions according to LUNGWORM-FL for the same period using meteorological data from the E-OBS gridded dataset.

5.2.3 Summary

LUNGWORM-FL is an adaptation of the previous GLOWORM-FL model (Rose et al., 2015) where lungworm specific parameters have been developed through the use of the available literature and design of laboratory studies. Notably, the addition of the role of the *Pilobolus* spp. to the model has enhanced the migration parameters to capture the significant role that the fungus has on lungworm epidemiology. Parameter estimates within faeces have been favourably compared to published studies. Migratory parameters are less well matched than survival and development in the faeces. The model predicted that more larvae would migrate onto the pasture during the summer than was found in the published studies although this could reflect the low recovery rate of larvae on herbage (Jørgensen, 1975b). The model was less accurate at predicting migration during the winter although this was less critical since cattle would be housed during this period and therefore at less risk from lungworm disease.

5.3 LUNGWORM-FL validation

5.3.1 Methods

5.3.1.1 Validation of the timing of peak pasture infectivity

This study was reviewed and approved by the University of Liverpool Veterinary Research Ethics committee (VREC 431).

Twelve dairy farms were recruited from across the UK with a history of lungworm disease within the past five years. A questionnaire was sent to participating farmers to gather information on herd size and dates of turnout and housing. The study ran from April to October in 2016 and 2017. Bulk tank milk samples were collected from the National Milk Records (NMR) laboratory on a fortnightly basis unless this coincided with farm testing by NMR, which meant that milk samples were unavailable for scientific use.

Bulk tank milk samples were defatted by centrifuging at 2,000g for 15 minutes before collecting the supernatant. During 2016, bulk tank milk samples were tested using a prototype of an ELISA plate developed by Boehringer-Ingelheim Svanova (Uppsala). For full details of milk processing and the ELISA performance, see Chapter 3 (although for model validation bulk tank milk samples rather than pooled – milk heifer samples were tested). Samples from 2017 were tested using a commercial ELISA from the University of Hannover, Germany (Schunn et al., 2012). The choice of kits was based on availability at the time. Both tests detect antibodies against the major sperm protein (MSP) for *D.viviparus*. Although the positivity cut-off varies between the two tests, this bears no influence on the longitudinal trends of interest.

The model was run for each farm's grazing season using meteorological data gathered by the E-OBS dataset (Haylock et al., 2008). During pilot experiments, faecal larval output was modelled at a set rate multiplied by the number of cattle in the herd. However, this was found to be an inaccurate method for determining the timing of seropositivity as it failed to account for any host component to the lifecycle. Therefore, faecal patency were based on the detailed faecal larval counts described by Eysker, Saatkamp and Kloosterman, (1993) using each herd's date of turnout and housing and multiplied by the number of grazing cattle per farm. The lungworm antibody titre is independent of infection dose (Strube et al., 2017) and so the model could not be used to directly predict antibody levels. However, the rise in antibody levels during a reinfection (the typical scenario for adult cattle) occurs between 36 and 46 days after peak larval pasture counts (Strube et al., 2017). Therefore, the model output was assessed to see whether a peak larval pasture count was predicted to occur between 22 and 46 days prior to the measured peak in antibody levels. A minimum of 22 days was used to accommodate for the fortnightly sampling regime.

5.3.1.2 Validation of the intensity of infection pressure

The Veterinary Investigation Diagnosis Analysis (VIDA) database was analysed for diagnosed cases of lungworm amongst dairy herds from 1975 to 2014 (Chapter 2). This is a passive surveillance system, which lists rough geographical data for submissions. To test whether LUNGWORM-FL could reflect annual differences in disease intensity as recorded by the VIDA database, model simulations were run for three randomly selected locations in each Nomenclature of Territorial Units for Statistics level two (NUTS2) region in the United Kingdom from 1975 – 2014. During these simulations, where specific timings were less important, 100 larvae were deposited on a daily basis across the grazing season to allow for a system of continuous grazing without making assumptions about grazing behaviour, herd

sizes or hypobiotic rates. Average grazing season dates from the cross – sectional risk factor study in Chapter 4 were used such that date of housing, h , and turnout, t , was the 8th April and 15th October respectively. Therefore, the overwintering period was assumed to be from the 16th October in one year to the 7th April in the consecutive year.

The area under the curve (AUC) was calculated for each study site using a trapezoid function in R to estimate the annual infection pressure over the following time periods for year y : $S_Y(AUC)$ (AUC between t_y and h_y); $O_Y(AUC)$ (AUC between h_{y-1} and t_y), $SO_Y(AUC)$ (AUC between h_{y-1} and h_y); $SS_Y(AUC)$ (AUC between t_y and h_y plus t_{y-1} and h_{y-1}). The AUC for each time period was log – transformed to approximate to a parametric distribution and the mean for each of the VIDA regions was calculated. This was correlated with the number of cases recorded by the VIDA database per year using Pearson’s correlation.

5.3.2 Results

An example of a modelled output from a farm at the Central Veterinary Laboratories, Weybridge (lat/lon: 51.355°N, -0.496°E) where 100 larvae per day were deposited from the 1st January 2002 to the 31st December 2004 is shown in Figure 5-9. Figure shows typical seasonality of disease with L3 on pasture increasing from April to July in each year and decreasing from October to December. The model predicts very little over – winter survival of L3 on herbage. Numbers of L3 in faeces remain moderate to high throughout the year but may be an artefact of modelling a faecal larval count of 100 larvae per day.

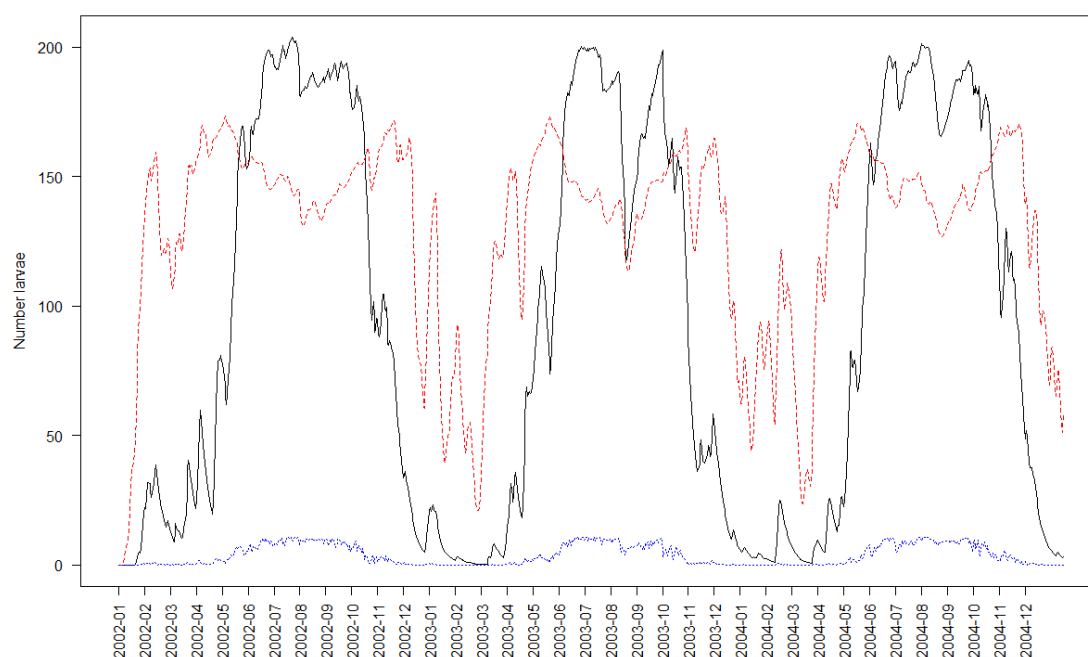


Figure 5-9: An example of output from the LUNGWORM – FL model showing pasture infectivity from the 1st January 2002 to the 31st December 2014. Faecal larval output assumed at 100 larvae per day. Figure shows the predicted number of L3 on herbage (black solid line), L3 in faeces (red hashed line) and L3 in soil (blue dotted line).

5.3.2.1 Validation of the timing of peak pasture infectivity

Eight farms displayed distinctive antibody peaks and were included in the validation of the model. Dates of turnout in 2016 ranged from the 25th March to 1st June whereas dates of housing ranged from the 7th September to the 30th November. During 2017, these dates were the 1st March to 20th March and the 1st November to 1st December respectively. The median length of the grazing season was 148 days in 2016 (25th – 75th quantile: 131, 174 days) and 250.5 days in 2017 (25th – 75th quantile: 238.3, 262.8 days).

The model predicted a peak in pasture infectivity that lay within the expected peak pasture infectivity boundaries in seven out of eight farms (Table 5.4 and Figure 5-10). Model output for farm 7 predicted a peak pasture infectivity occurring 8 days prior to the earliest expected day for peak pasture infectivity. According to LUNGWORM-FL, an antibody rise would have been expected to occur between the 4th and 14th July 2017. However, due to prior obligations with NMR, milk was unavailable for testing from farm 7 between the 30th April and 24th July 2017 and so it was possible that an earlier antibody peak was missed in the milk sampling.

One herd developed signs of a clinical outbreak of the disease during the study period. According to the LUNGWORM-FL output, farm 4 would expect to see a peak pasture infectivity on the 19th July 2016. This farm reported clinical signs of lungworm disease starting between 22 – 26 days after the model predicted pasture infectivity would be highest. Clinical signs can occur as early as 13 days post-infection in naïve calves (Parker, 1957) with faecal patency detected from 18-22 days post infection (Tenter et al., 1993). Therefore, the date of clinical onset of an outbreak in this herd fits with the model predictions.

Excluding farm 7, the model predicts that the average duration between peak pasture infectivity and peak antibody rise was 46 days (95% confidence intervals 38 – 52 days). Given the fortnightly sampling regime, this could be as early as 32 days (95% confidence intervals 24 – 38 days) post peak pasture infectivity.

Table 5.4 Comparison between expected peak pasture infectivity (22 - 46 days prior to antibody peak) and modelled peak pasture infectivity based on LUNGWORM-FL output.

Farm	Location	Grazing season (dd/mm/yy)		Antibody peak (dd/mm/yy)	Expected peak pasture infectivity (dd/mm/yy)		Modelled peak pasture infectivity (dd/mm/yy)	Duration between antibody peak and modelled peak pasture infectivity (days)
		Turnout	Housing		Min	Max		
2016:								
1	Southwest Scotland	30/04/16	07/09/16	24/07/16	25/05/16	18/06/16	16/06/16	38
2	Southwest Scotland	21/04/16	30/09/16	25/07/16	26/05/16	19/06/16	12/06/16	43
3	Northwest England	04/05/16	15/09/16	04/08/16	05/06/16	29/06/16	24/06/16	41
4	Northwest England	20/04/16	15/10/16	05/09/16	07/07/16	31/07/16	19/07/16	48
5	Midwest England	25/03/16	30/11/16	12/08/16	13/06/16	07/07/16	21/06/16	52
6	Midwest England	01/06/16	30/09/16	07/09/16	09/07/16	02/08/16	17/07/16	52
2017:								
7 *	Northwest England	01/03/17	01/12/17	05/08/17	06/06/17	30/06/17	29/05/17	68
8	Midwest England	20/03/17	01/11/17	04/08/17	05/07/17	29/06/17	16/06/17	49

* Modelled peak pasture infectivity outside of range of expected peak pasture infectivity

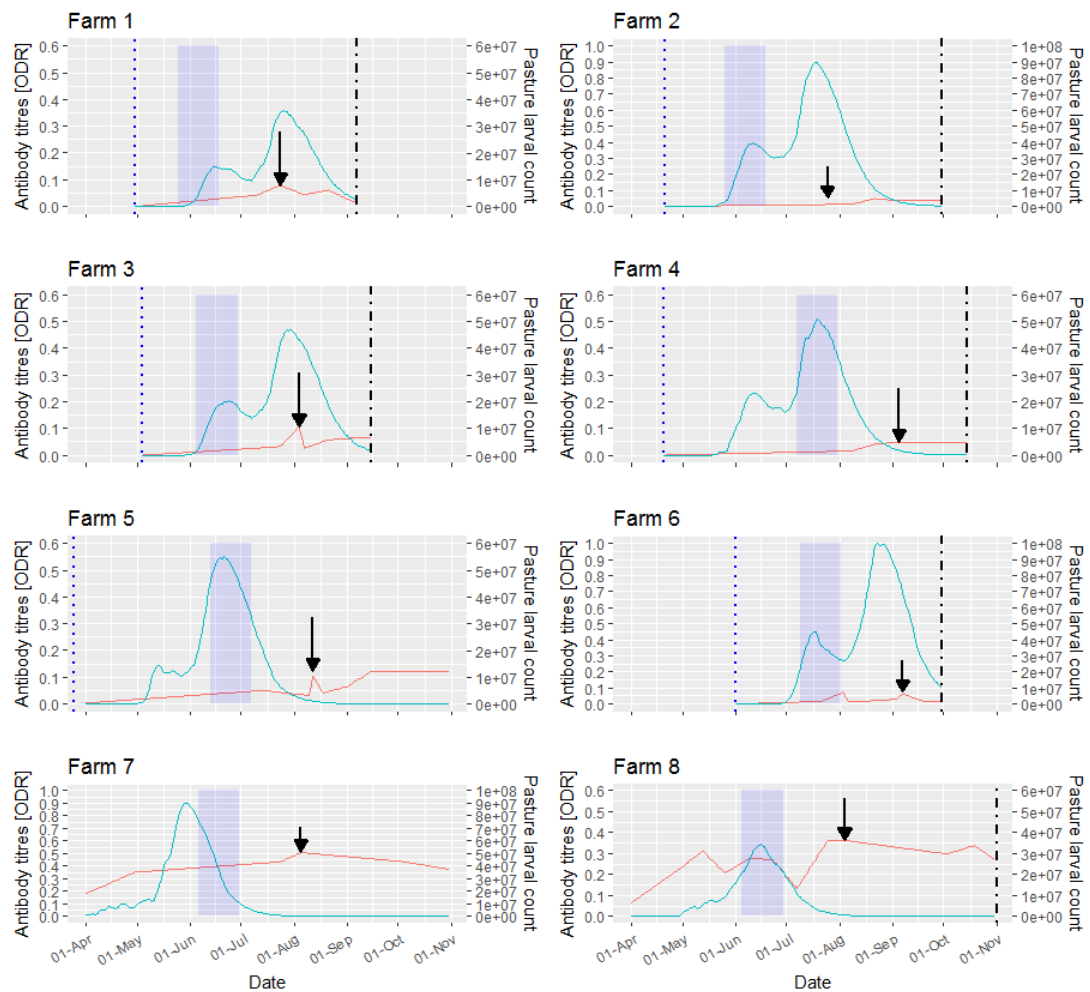


Figure 5-10: *Dictyocaulus viviparus* antibody titres (red line) in fortnightly bulk tank milk sampling of eight herds in Great Britain. Black arrows refer to peak antibody titres. Note that subtle antibody changes may not be directly visible given y-axis scale. Dates of turnout (blue dotted line) and dates of housing (black hashed line) shown although for simplicity, x-axis has been limited between 1st April and 1st November. Data from 2016 (farms 1-6) and 2017 (farms 7-8). Purple shaded regions refer to expected peak pasture infectivity based on date of peak antibody titre (black arrow). Number of infective L3 larvae present on herbage according to LUNGWORM-FL model output (green line). Note the different y-axis scale for farms 2, 6 and 7.

5.3.2.2 Validation of the intensity of infection pressure

The area under the curve during the current grazing season, $S_y(AUC)$, predicted between 0.0 – 24.4% of the annual variations in disease intensity depending on geographical location (Table 5.5 and Figure 5-11). There was a moderate correlation in Scotland ($r=0.49$, $p<0.001$), Wales ($r=0.49$, $p<0.001$), midwest England ($r=0.41$, $p=0.01$), northern England ($r=0.39$, $p=0.01$) and southwest England ($r=0.33$, $p=0.04$). Model predictions were not significantly correlated to disease frequency measures in the east and southeast of England ($r=0.0$ and 0.28 , $p=0.98$ and 0.07 respectively). When east and southeast England were excluded, the model predicted between 10.7 – 24.4% annual variations in disease intensity as recorded by the VIDA database. The area under the curve during the overwintering period, $O_y(AUC)$, predicted less of the annual differences in lungworm cases, explaining only 0.4 – 7.2% of the variation between years. No region showed a significant correlation between model predictions during the overwintering period and number of cases. The addition of the previous year's grazing season, $SS_y(AUC)$, did not enhance the correlation except in Wales which showed a strong correlation ($r=0.52$, $p<0.001$) explaining 27.5% variation between years.

Table 5.5 Correlation between log-transformed area under curve predicted by the LUNGWORM-FL model and the number of lungworm cases diagnosed by VIDA database from 1975 – 2014.

Region		$S_y(AUC)^a$			$O_y(AUC)^a$			$SO_y(AUC)^a$			$SS_y(AUC)^a$		
		r	$R^2(\%)$	p	r	$R^2(\%)$	p	r	$R^2(\%)$	p	r	$R^2(\%)$	p
England	North	0.39	15.4	0.01*	0.20	4.0	0.22	0.37	13.6	0.02*	0.37	13.4	0.02*
	Midwest	0.41	17.2	0.01*	0.21	4.4	0.19	0.37	13.8	0.02*	0.40	16.0	0.01*
	East	0.00	0.00	0.98	-0.11	1.2	0.50	-0.09	0.8	0.59	-0.16	2.6	0.32
	Southeast	0.28	8.0	0.07	0.06	0.4	0.69	0.23	5.4	0.15	0.21	4.2	0.20
	Southwest	0.33	10.7	0.04*	0.16	2.4	0.33	0.30	8.8	0.06	0.31	9.4	0.05
Scotland		0.49	24.4	0.00***	0.09	0.8	0.58	0.42	18.0	0.01*	0.38	14.4	0.02*
Wales		0.49	24.2	0.00***	0.27	7.2	0.09	0.48	22.6	0.00***	0.52	27.5	0.00***
UK		0.15	2.2	0.01*	0.07	0.51	0.23	0.12	1.4	0.05	0.11	1.2	0.07

^a Area under curve (AUC) modelled during current grazing season (S_y), previous overwintering (O_y), previous overwintering plus current summer (SO_y) and previous plus current summer (SS_y).

Significance: * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

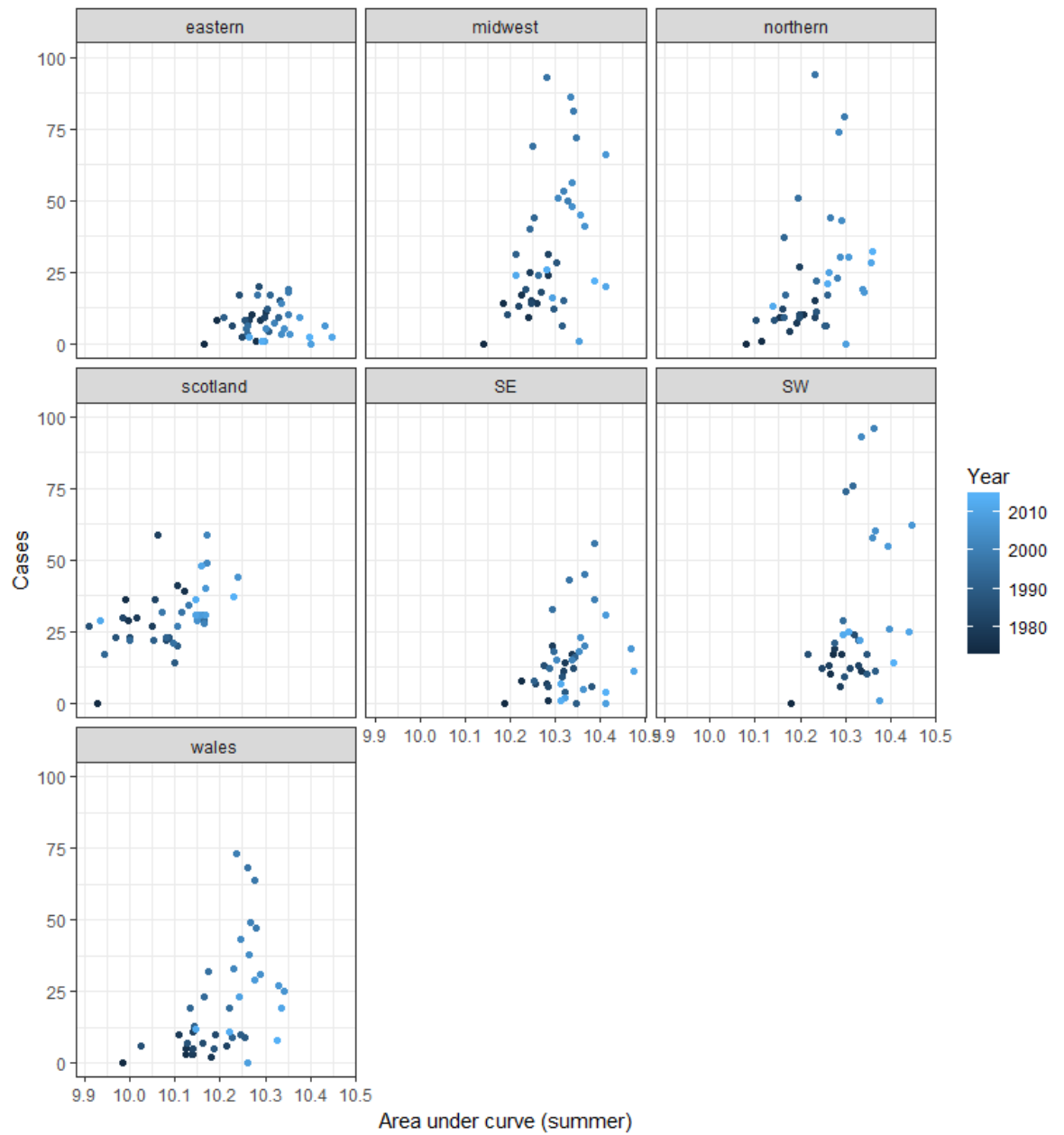


Figure 5-11: Correlations between log-transformed areas under curve during the grazing season as predicted by LUNGWORM-FL model by number of cases recorded in VIDA database in each region of Great Britain. Colours refer to years from 1975 to 2014.

5.4 Summary

LUNGWORM-FL predicted the timing for peak pasture infectivity in seven out of eight dairy herds. The herd that could not be predicted had an absence of milk available for testing during the first half of the grazing season and so it was probable that an antibody peak was missed. Furthermore, one herd developed clinical signs of a lungworm outbreak during the study period. The model was able to predict that peak pasture infectivity could occur between 22 – 26 days prior to the onset of clinical signs which coincides with expected faecal output timings (Tenter et al., 1993). The average duration between peak pasture infectivity and peak antibody titres was 46 days (95% confidence intervals 38 – 52 days). This coincides with the latest cattle studies which found that the rise in antibody levels during a reinfection occurs between 36 – 46 days after peak larval pasture counts (Strube et al., 2017).

There was a moderate correlation between model predictions and annual differences in disease intensity according to the VIDA passive surveillance system reports. In regions with high cattle density (Scotland, Wales, midwest -, northern - and southwest England), between 10.7 – 24.4% annual variation could be predicted by the area under the curve (AUC) in the current grazing season. The model was unable to predict significant annual differences in disease intensity in east and southeast England. The addition of AUC during the overwintering period and the previous summer did not enhance model predictions except for the addition of the previous grazing season to the predictions in Wales, which predicted 27.5% variation between years.

5.4 Model application under historic and future climate scenarios

5.4.1 Methods

To understand the changing infection pressure under both short and long-term future climate change predictions, the validated LUNGWORM-FL model was run using predicted temperature and rainfall data for 1st January 2024 – 31st December 2099. Mean daily near surface air temperature and total daily precipitation data were collected from the atmospheric dataset provided by the Coupled Model Intercomparison Project Phase 5 (CMIP5; Taylor et al., 2012) using a high emissions scenario (Representative Concentration Pathway 8.5; RCP8.5). This used the HadGEM2-ES model output (ensemble r1i1p1, version 20111128), developed and run by the Met Office Hadley Centre (Collins et al., 2011; Martin et al., 2011).

The same three randomly selected study locations per NUTS2 territory were selected as those used to validate the infection pressure from 1975 – 2014 (see Section 5.3.1.2). During these simulations, 100 new larvae were deposited on the pasture during the grazing season (defined as 8th April – 15th October) to simulate cattle grazing. The model was allowed to progress continuously throughout the 75 years and any larvae that survived overwinter were accommodated into the next year's grazing season. Therefore, the first year of simulations (2024) was removed from analysis.

Infection pressure was calculated as the natural logarithm of the area under the curve (AUC) during the grazing season (8th April to 15th October) as this had most correlation with the number of lungworm cases reported in the VIDA database during model validation. Annual

infection pressures from 1975 to 2014 were calculated and divided into quantiles to create an infection pressure percentile. The infection pressures during the predicted climate change scenarios were compared with the infection pressure percentiles from 1975 – 2014.

Historic (1975-2014) and future (2025-2099) infection pressure and temperature differences were normally distributed and compared using the mean, standard deviation and t – test. Rainfall data was not normally distributed and so was compared using the median, 25% and 75% quantiles and the Mann – Whitney U test.

5.4.2 Results

5.4.2.1 Historic trends (1975 – 2015)

The UK average infection pressure rose from 1975 (log AUC 10.20) to peak in 2009 (log AUC 10.31) (Figure 5-12c). From 2005 - 2015, the infection pressure rose to its predicted highest, with 80-100% quantile evident throughout the majority of England (Figure 5-12a). According to LUNGWORM-FL predictions, during the 1970s and 1980s, Scotland and northern England had a climate that was unsuitable for *D.viviparus* transmission on pasture. However, from 1995 onwards, meteorological conditions in northern England and southwest Scotland were such that *D.viviparus* transmission became possible. This coincided with a significant increase in the daily temperatures in Scotland from 1985 – 1989 to 1995 – 1999 (mean $11.4 \pm 3.2^{\circ}\text{C}$ and $12.2 \pm 3.5^{\circ}\text{C}$ respectively, $t(1895.6)=5.50$, $p<0.001$) and a borderline significant increase in rainfall (median 0.4 mm (0.0-4.1mm) and 1.0 mm (0.0-4.6mm) respectively, $W=433940$, $p=0.05$).

5.4.2.2 Short-term future predictions (2025 - 2045)

The model predicts a small but significant decrease in the infection pressure across the UK between historic (1975 – 2015) and short-term (2025 – 2045) climate trends (mean 10.26 ± 0.12 and mean 10.25 ± 0.11 respectively, $t(6312.1)=4.00$, $p<0.001$) (Figure 5-12b). By 2037, the infection pressure in Cheshire will be below the baseline 1975 level. Infection pressure in Cornwall is predicted to peak from 2014 – 2030 (log AUC 10.42) and thereafter decrease slightly to the end of the simulations in 2099 (log AUC 10.26). Nonetheless, Cornwall is predicted to remain the region with the highest infection pressure in both the historic and future predictions. The timing for the decreased infection pressure from 2030 in Cornwall coincides with a significant increase in daily temperature during the grazing season (2020 – 2024: mean $15.9 \pm 3.1^{\circ}\text{C}$; 2030 – 2034: mean $16.3 \pm 3.3^{\circ}\text{C}$; $t(1646.6)=-2.9$, $p=0.004$). This

was not associated with a significant difference in rainfall (2020 – 2024: median 0.19 mm (0.00 – 0.98); 2030 – 2034: median 0.16 mm (0.00 – 0.80); $W=370570$, $p=0.08$).

The infection pressure in southwest Scotland was predicted to be below the UK average in 1975 but by 2021, it is predicted to exceed Cheshire's baseline levels and be above the UK average by 2025. The infection pressure is predicted to peak in the years 2032 – 2051 with a significant rise in infection pressure between the historic and short-term future predictions (mean 10.2 ± 0.09 and 10.3 ± 0.05 respectively, $t(195.3)=-14.6$, $p<0.001$).

5.4.2.3 Long-term future predictions (2045 – 2099)

The overall UK infection pressure is predicted to decrease until the end of the simulations in 2099 (log AUC 10.10) such that by 2070, the UK average infection pressure is predicted to be below baseline levels in 1975 (Figure 5-12c). The difference in infection pressure between historic and long-term future predictions was significant (mean 10.26 ± 0.12 and mean 10.09 ± 0.17 respectively, $t(10415)=59.1$, $p<0.001$) By 2055, the majority of the UK, excluding Scotland and southwest England, is predicted to have a climate which is unsupportive of *D.viviparus* transmission on pasture (Figure 5-12b).

Infection pressures in Scotland are predicted to peak in 2052 and thereafter decrease. This decrease is associated with a significant increase in daily temperatures (2042 – 2046: mean $15.0 \pm 4.3^{\circ}\text{C}$; 2052 – 2056: mean $15.5 \pm 4.1^{\circ}\text{C}$; $t(1876.1)=-2.7$, $p=0.01$) with no significant change in rainfall (2042 – 2046: median 0.67 mm (0.04 – 2.72); 2052 – 2056: median 0.55 mm (0.03 – 2.48); $W=454780$, $p=0.27$).

By 2090, there is not predicted to be a significant difference in the infection pressure between Scotland and Cornwall (mean 10.2 ± 0.11 and 10.3 ± 0.05 respectively, $t(35.9)=1.9$, $p=0.06$). These regions are both predicted to have a significantly higher infection pressure than in Cheshire (Cheshire mean 10.0 ± 0.12 , comparison Cheshire and Scotland $t(51.4)=-6.9$, $p<0.001$; or Cornwall $t(34.1)=10.3$, $p<0.001$). By 2090 – 2094, temperatures in Cheshire ($20.0 \pm 5.4^{\circ}\text{C}$) are significantly warmer than in either Scotland ($18.1 \pm 4.9^{\circ}\text{C}$; $t(1859)=-7.7$, $p<0.001$) or Cornwall ($18.4 \pm 3.6^{\circ}\text{C}$; $t(1643.8)=-7.5$, $p<0.001$).

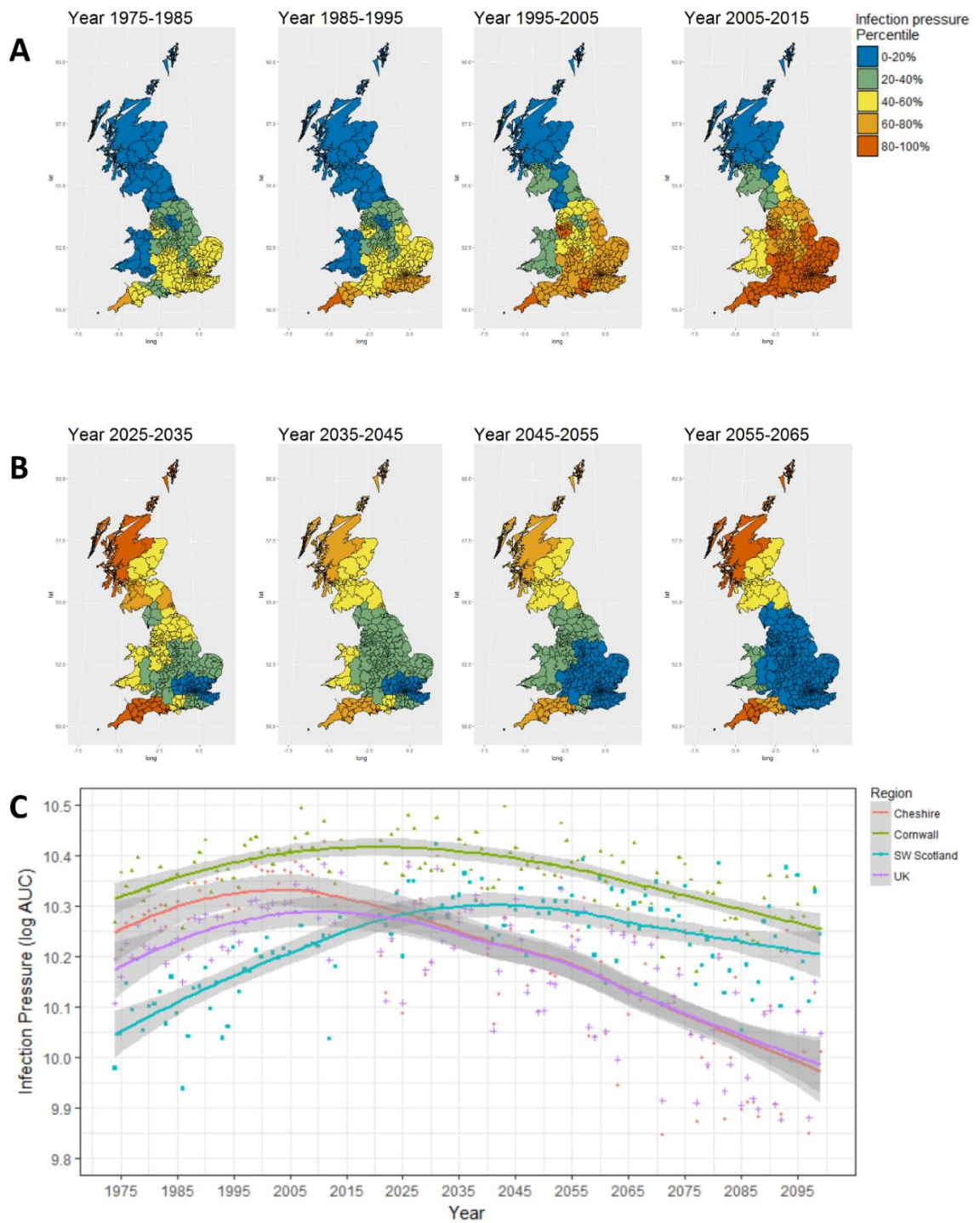


Figure 5-12: *Dictyocaulus viviparus* infection pressure (natural logarithm of the area under curve from 8th April to 15th October) as predicted by the LUNGWORM-FL model a) under historic climate conditions (1975 to 2015) or b) in future climate change conditions (2025 – 2065) using three randomly selected locations per NUTS 2 territory. Region specific infection pressures are shown in c) for, Cheshire (red circles), Cornwall (green triangles) and southwest Scotland (blue squares) from 1975 - 2099. Trend lines show LOESS smoothing with 95% confidence intervals of trend line.

5.4.3 Summary

According to LUNGWORM-FL predictions, the average UK infection pressure rose from 1975 (log AUC 10.20) to peak in 2009 (log AUC 10.31) and will thereafter decrease until the end of the simulations in 2099 (log AUC 10.10). The exception to this rule is in Scotland, which is predicted to see an increased risk from lungworm disease until 2051 with pressures exceeding the national average by 2025. By 2090, the infection pressure in Scotland is predicted to equal that of Cornwall.

Changes in lungworm epidemiology have been closely associated with changes in daily temperatures during the grazing season. An increase from $11.4 \pm 3.2^{\circ}\text{C}$ to $12.2 \pm 3.5^{\circ}\text{C}$ was associated with an increased infection pressure in Scotland from 1995 onwards. The infection pressure is predicted to decrease in Cornwall by 2030 when temperatures increase from $15.9 \pm 3.1^{\circ}\text{C}$ to $16.3 \pm 3.3^{\circ}\text{C}$ and in Scotland by 2052 when temperatures increase from $15.0 \pm 4.3^{\circ}\text{C}$ to $15.5 \pm 4.1^{\circ}\text{C}$. Rainfall changes were not associated with infection pressure changes.

5.5 Discussion

LUNGWORM-FL is the first validated model of the free-living stages of the life cycle of *D. viviparus*. It proved accurate in depicting seasonal rises in infection pressure in the first half of the grazing season and decreases during the autumn. It accurately predicted the timing of peak pasture infectivity in seven out of eight herds and predicted specific years for the increased spatial distribution of the disease into Scotland. However, the model has several key assumptions, which have been transparently declared. It also only models the free – living stages and so cannot incorporate changes in host – parasite interactions, such as altered grazing season lengths, which may occur under future climate change scenarios. Further work is needed to test some of the assumptions from this model and to continue to produce validation data for the parameters and the model output.

After further validation, this model would function as a predictive tool for farmers and veterinary surgeons who could alter grazing practices or apply anthelmintics when the risk in a particular area is seen to increase. Such parasite forecasting systems have widely been adopted to alert the farming community to an increased infection risk of parasitic gastroenteritis, *Nematodirus battus* in sheep and *Fasciola hepatica* in sheep and cattle (NADIS, 2018). In addition, the model could help to enhance the diagnostic capability during a lungworm outbreak. According to LUNGWORM-FL, a rise in antibody titres occurs 46 days (95% confidence intervals 38 – 52 days) after a peak pasture infectivity. This is in agreement with recent studies which found that, during reinfections, the antibody response occurs 36 – 46 days after peak larval pasture counts (Strube et al., 2017). The first clinical signs of lungworm disease, can be seen from 13 days post-infection onwards (Parker, 1957). The ability to diagnose lungworm during early clinical outbreaks is limited, with neither faecal patency nor antibody titres becoming positive until 23 – 28 days and 30 – 32 days post-

infection respectively (Fiedor et al., 2009). However, the model predicted a peak in pasture infectivity occurring 22 – 26 days prior to the development of clinical signs. The model could potentially be used as a quick and cheap tool to alter the index of suspicion prior to more formal diagnostics becoming positive. More work is needed to understand the practical implications of using the model for this purpose.

In regions of the UK with the highest cattle density, the model predicted 10.7 – 24.4% of the annual variation in the number of clinical cases reported to the Veterinary Investigation Diagnosis Analysis (VIDA) database between 1975 – 2014. The VIDA database has several major limitations in its use as a model validation resource. It is a passive surveillance system and, for example, farmers who are unwilling to pay for diagnostic tests will not contribute to the recorded number of cases. It is possible that there are regional differences in reporting behaviour. Farmers in southwest England for example, who regularly see lungworm cases, may be less likely to pay for cases to be diagnosed by the regional laboratories. An accurate assessment of the model's ability to predict annual differences is unlikely to occur without more detailed geospatial case data. Further work is needed to understand the model's functionality in the future. A more optimal strategy would be to recruit several sentinel farms from across Great Britain to collect longitudinal data of parasite burden, such as the system proposed in Belgium (Charlier et al., 2016b).

Annual differences were best estimated from the infection pressure during the current grazing season only, and not through the addition of the infection pressure during the overwintering period, or previous grazing season. This suggests that the degree to which larvae manage to survive over winter at pasture does not play a significant role in the likelihood of disease in the next year. This is in agreement with studies suggesting that if

overwintering is possible, this will only happen in low numbers for lungworm larvae (Eysker et al., 1994b; Hertzberg et al., 1996; Jørgensen, 1980a; Oakley, 1982). The only exception to these findings was Wales where there was an improvement to the model predictions when the previous year's grazing season was added. The presence of lentic water bodies has been shown to be a significant risk factor for lungworm disease (Schunn *et al.*, 2013, Chapter 4). It is possible that Wales, with its comparatively boggy climate, has a greater potential for overwintering survival than other parts of the UK.

The model was parameterised using a combination of data from published literature sources and purposefully designed laboratory studies. Both techniques for model parameterisation offer unique benefits. Using published literature enables a broader range of conditions to be explored with access to a larger number of data points and the use of several operators will reduce operator error. Conversely, laboratory studies can be specifically designed to fill knowledge gaps, the methodology is clear and studies can be internally validated. The model parameters showed a strong correlation to independent studies monitoring larval populations developing in the faeces during the summer and the winter. The recovery rate for *D. viviparus* larvae on herbage has been found to range from 33.3 – 91.9% (Jørgensen, 1981). The lower model fit for larval recovery on pasture during the winter was not seen as a major obstacle to the accuracy of model output since cattle are housed during the winter but should be accurately measured in future laboratory studies.

The historic trends predicted by LUNGWORM-FL agree with surveillance reports from 1975 – 2015 (Chapter 2). The model predicts that the infection pressure across the UK has increased annually since 1975 with 80 – 100% quantile evident throughout the majority of the UK by 1995 – 2015. This effect was most apparent in Scotland, with an increased infection

pressure beginning in 1995 and increasing to exceed the UK average by 2025. This agrees with reports indicating that there was a significant increase in cases of lungworm reported to the VIDA database during the 1990s (David, 1999) and a further increase in cases reported in 2014 according to the Great Britain Cattle Disease Surveillance emerging threats quarterly report (Department for Environment Food and Rural Affairs et al., 2014). Furthermore, there were twice as many cases of lungworm diagnosed across Scotland in 2010 (n=12) than in 2009 (n=6) (Veterinary Services, 2010). The degree to which the changing spatiotemporal distribution of lungworm can be modelled by a climate – based mathematical model supports the notion that climate changes have already had a major influence on the epidemiology of this parasite.

Any changes predicted during future climate change scenarios are based on the best estimate of climate change predictions from the RCP 8.5 scenario (Taylor et al., 2012; van Vuuren et al., 2011). Dates and years predicted are based on climate change forecasts that have the potential to change if global changes are put in place to halt the development of climate change. However, the model can quantify the degree to which the temperature would need to change to start to see epidemiological changes. There appears to be a narrow window that is able to facilitate optimal transmission on pasture. LUNGWORM-FL predicted that the infection potential would raise in Scotland when mean temperatures from 8th April – 15th October increased from $11.4 \pm 3.2^{\circ}\text{C}$ to $12.2 \pm 3.5^{\circ}\text{C}$. However, the infection pressure is predicted to decrease in Cornwall once mean temperature reaches $16.3 \pm 3.3^{\circ}\text{C}$ and in Scotland when it reaches $15.5 \pm 4.1^{\circ}\text{C}$. A higher proportion of first year grazing cattle herds were found to be serologically positive against *D.viviparus* if there were at least 50 days between May and September with an average daily temperature exceeding 15°C (Schnieder et al., 1993). This suggests that lungworm epidemiology has a narrow optimum temperature

range between 12.2 – 16.3°C. The average UK temperatures during the summer months (June – August) from 2014 to 2017 was 14.6°C (Met Office, 2018). It is interesting therefore that the model predicts that the UK is currently at the height of lungworm intensity. However, studies from Malaysia (Lat-Lat et al., 2010), Tanzania (Thamsborg et al., 1998) and Costa Rica (Jiménez et al., 2007) suggest that transmission still occurs outside of modelled temperature windows even if transmission rates are not optimal. Therefore, it is important to note that ‘suboptimal’ transmission may still lead to clinical disease. Future model work should explore whether the between-year variability in the timing of disease will increase under future climate change scenarios. Disease may occur more sporadic and more unpredictable, making it even more devastating in dairy herds. This may pose unique challenges to the maintenance of immunity within a herd. Future studies should combine the free – living with a parasitic model to provide more precise predictions of overall future disease intensity and suggest protocols to counteract these changes.

Although the model predicts that the overall UK infection pressure will decrease under future climate change scenarios, the southwest England and Scotland will remain at a high risk from the disease. Frequently purchasing cattle is a significant risk factor for lungworm entry onto a farm (Charlier et al., 2016a). If the UK infection pressure decreases to the geographical extent that is predicted by the model, the majority of England’s dairy herd are at risk of becoming serologically naïve to the disease. This could be a potentially disastrous situation if cattle from southwest England or Scotland are brought onto an English farm. Therefore, despite the decreased overall UK risk, explosive outbreaks could still occur after imported animals enter the herd. For this reason, it remains fundamental that farmers either maintain an active immunity against the disease, for example, using the vaccine, or conduct strict biosecurity measures when animals enter the herd.

The model predictions during future climate change scenarios assume that there will be no evolutionary adaptation by either *D.viviparus* or *Pilobolus* spp. to a changing climate. The model suggests that increases in daily temperatures would increase the instantaneous daily development rate for larvae. However, preinfective larval stages are sensitive to high temperatures (Daubney, 1920) with the model suggesting that by 22.5°C, mortality rates of L1 and L2 (μ_1 , μ_2) have increased from 0.2 to 0.3/day. Although the development and mortality rates of gastrointestinal nematodes of sheep have been shown to not be highly adaptable under climate change scenarios, other traits such as the onset of hypobiosis are more adaptable (van Dijk et al., 2010). Hypobiosis is an evolutionarily expensive trait, reducing the basic reproductive rate (R_0) of trichostrongylid nematodes (Dobson and Hudson, 1992) and having a destabilising effect on the host-parasite interactions (Gaba and Gourbière, 2008). Therefore, it could be predicted that if future climate change scenarios are such that overwinter survival is more conducive than in the present epidemiology, then hypobiosis will become less favoured under evolution adaptations. The model analysis has assumed that $S_Y(AUC)$ remains the best indicator for disease intensity under climate change scenarios. However, by allowing the model to progress unhindered from 2024 – 2099, any changes in overwintering ability will have been incorporated into the results. Furthermore, work presented here may serve as a useful base point in which to refer any future adaptations of the parasite against.

5.6 Conclusion

LUNGWORM-FL is the first validated model of the free – living stages of the bovine lungworm, *D.viviparus*. It has been parameterised through data available in the literature and the development of laboratory studies. The model can predict a peak in pasture infectivity which occurs 46 days (95% confidence intervals 38 – 52 days) prior to a rise in antibody levels and 22 – 26 days prior to the onset of clinical signs. This model has the ability to function as a disease awareness tool to alert farmers and vets to the risk of lungworm in an area.

The model predicts that lungworm epidemiology is optimised in temperatures between 12.2 – 16.3°C. According to the model output, once climate change is sufficient to bring average temperatures beyond this range, the overall UK infection pressure will decrease. This could occur as early as 2025 if current climate change forecasts are reliable. The risk in Scotland and southwest England are predicted to remain high throughout the short- and long-term future. Cattle entering herds from these endemic areas could lead to an explosive disease outbreak in an otherwise naïve herd. Therefore, vigilance and maintenance of immunity despite a perceived reduction in risk is of utmost importance.

5.7 Acknowledgements

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Chapter 6: General discussion

6.1 Summary of results and future directions

Dictyocaulus viviparus has proven to be a fascinating parasite to study from an epidemiological perspective. The changes observed in the spatiotemporal distribution and case demographics suggest an evolving disease process, which is insufficiently controlled by current farm management practices. A key finding has been that, in sharp contrast with what has been proposed in the past, the epidemiology of the parasite seems to be driven primarily by climatic conditions. Previous workers have invariably looked towards farm-management practices as key risk factors for lungworm disease. As climate cannot be manipulated and current control appears to be failing in many cases, this appears to be a reason for concern. The climate – driven increase in prevalence is even more of a concern when considered within the current parasitological challenges of the emergence of anthelmintic resistance, altered farm management practices and increasing environmental and economic demands upon farmers.

The numerous anecdotal reports from farmers and veterinarians [personal communication acquired as part of the PhD] together with crude analysis and surveillance reports (David, 1999; Department for Environment Food and Rural Affairs et al., 2014; van Dijk, 2004; Veterinary Services, 2010), suggested that lungworm disease is re-emerging across Great Britain, with the farming community in northern England and Scotland particularly struggling to control the disease. This was confirmed by the analysis of the Veterinary Investigation Diagnosis Analysis (VIDA) dataset in Chapter 2 which found that there was a four – fold increase in diagnostic rates across Great Britain from 1980 (0.97 cases per 1,000 submissions)

to 2014 (4.04 cases per 1,000 submissions). This was characterised by a dramatic increase from 1995 to 2000 and a significant but less extreme decline from 2000 to 2014. Parasitic gastroenteritis rates in cattle also increased from 2.06 cases per 1,000 submissions in 1990 – 1999 to 4.71 cases per 1,000 submissions in 2000 – 2004. Similar increased rates across Great Britain have been reported for parasitic gastroenteritis in sheep (van Dijk et al., 2008) and *Fasciola hepatica* infection in cattle and sheep (van Dijk et al., 2010). The overall trend of a decrease in *D.viviparus* diagnostic rates from 2004 was mainly due to a significant decrease in cases diagnosed from southwest and east England and may reflect a complacency in reporting cases, particularly in southwest England, which regularly saw cases of lungworm pre – 2004. The disease has spread northerly, with Scotland seeing fewer than 10% of the cases in 1999 to becoming the region with the highest proportion of cases across the country by 2009. Moreover, there has been a change in the seasonality of the disease with a higher proportion of cases diagnosed during the winter in southwest England and Wales.

Strikingly, the LUNGWORM-FL model outputs captured the changing spatiotemporal distribution of lungworm disease since the mid-1990s entirely. The model predicted that the majority of Great Britain, including northern England and Scotland, had a climate which was increasingly conducive to the development and transmission of *D.viviparus* larvae on pasture from 1995 (Chapter 5). The addition of the fungus *Pilobolus* spp. to the model added an additional level of complexity to the migration parameters, which accounted for the rapid translation of larvae from faeces to herbage once the larvae had reached the infective stage. The model predicted that the infection pressure began to increase in Scotland from 1995 once the mean daily temperature increased from $11.4 \pm 3.2^{\circ}\text{C}$ to $12.2 \pm 3.5^{\circ}\text{C}$. The threat of disease is predicted to remain high in Scotland until mean daily temperatures reach $15.5 \pm 4.1^{\circ}\text{C}$, predicted by 2052 according to the RCP 8.5 scenario. Laboratory experiments on the

free – living stages suggested that these subtle temperature changes will be associated with a more rapid development rate for larvae offset by a higher mortality rate of the first (L1) and second (L2) stage larvae in faeces. In the short – term, these trends may continue. What the model does not capture is any future evolutionary adaptations that the parasite or *Pilobolus* spp. fungus may undergo under climatic stress. It is currently thought that lungworm larvae have a poor ability to overwinter on pasture and that most of the year to year survival of the parasite is due to hypobiosis in carrier animals (Eysker et al., 1994a; Saatkamp et al., 1994). However, if the climate is such that there is a prolonged survival of larvae in faeces or on pasture during winter, and temperatures are such that development opportunities occur earlier in the year, then larvae may become less reliant on hypobiosis and increase their survival on pasture over – winter. Similar trends with an increased infection pressure in the winter and decreased pattern in the late summer are predicted to occur for the gastrointestinal nematodes of cattle under climate change scenarios (Rose et al., 2016). Worryingly, the altered seasonality and increase in cases diagnosed during the winter from the VIDA database (Chapter 2) may suggest that these changes are already happening in *D.viviparus* isolates from the southwest England and Wales. Future laboratory studies are needed to assess current differences in isolates between different parts of Great Britain.

The model also cannot capture differences in host – parasite interactions under future changes. The grazing season length is predicted to increase by up to 2.5 months in northern - eastern Europe under climate change scenarios (Phelan et al., 2016). What is missing from these predictive models is any integration of pasture conditions into the model. For example, grass growth under high temperatures, high ultraviolet light and reduced precipitation may be poor. This could mean that farmers are forced to supplement cattle roughage during the

driest, hottest months. This could encourage farmers to alter their grazing strategies, including either grazing cattle for shorter periods, or even grazing cattle in a biphasic fashion during the spring and autumn but not the summer months. Current models oversimplify grazing length based on temperature and rainfall conditions. Future climate change forecasts should integrate free – living parasite models with parasitic host - stage models and models predicting grass growth, to provide an accurate assessment of the impact that climate change will have on parasite infection pressures.

Theoretically, under climate change scenarios, cattle from different geographical regions may harbour parasite burdens that are adapted to different climates meaning that disease patterns may alter depending on where cattle are imported. Moreover, there will be unique challenges in the maintenance of herd immunity under climate change scenarios. Although the risk of disease across Great Britain is predicted to decrease within the next 10 – 20 years, significant hotspots for disease will remain in southwest England and Scotland. Therefore, there is a significant risk that immunity levels will fall across the majority of England, risking an explosive outbreak if cattle are imported without proper quarantine and anthelmintic treatments from one of the higher – risk areas. If this coincides with the emergence of anthelmintic resistant *D.viviparus* isolates, this could develop into a dangerous, expensive and devastating outbreak. Methods of sustainable nematode control have led to some encouraging results in sheep but initial attempts for lungworm control in cattle have been disappointing (Höglund, 2006; Ploeger and Holzhauer, 2012) and further work is needed in this field.

There has been an increase in cases of lungworm diagnosed in adult cattle aged 2 years and above since the 1980s (Chapter 2). Anthelmintics were given more than twice a year to 29%

cows in the cross – sectional study of grazing dairy herds in Great Britain with 62% of farms not using the lungworm vaccination (Chapter 4). Several detailed studies exist which look at the development of immunity to *D.viviparus* whilst cattle are being treated with both short and long - acting anthelmintics (see for example Bonazzi *et al.*, 1983; Jacobs *et al.*, 1989; Eysker *et al.*, 1995; Grimshaw *et al.*, 1996; Schnieder *et al.*, 1996; Taylor *et al.*, 2000). Despite this variety of research, long – acting anthelmintics are commonly used which provide the least opportunities to develop a strong immunity. Strong incentives will need to be provided in order to change to a targeted anthelmintic policy rather than blanket treatment. Further useful research would be to perform a systematic review to understand the degree to which cattle can develop immunity whilst under different anthelmintic treatments. Alternatively, mathematical models should be used to understand the trade – offs between development of immunity, production losses and the potential impact on milk withdrawal periods. Ideally, cattle studies should be performed to confirm that the changes suggested by the modelling and review approach are replicated under field conditions.

Significant farm – management factors associated with an increased pooled – milk antibody level were the presence of a water body and grazing cattle away from the main herd (Chapter 4). This study suggested that more research is needed to understand the infection dynamics when heifers join the main milking herd, including when they return to the farm after grazing away. The sensitivity and specificity of farmer’s impression on whether lungworm was present on the farm was poor. More frequent testing of herds should become routine as part of herd – health plans. The cheap pooled – milk test described in Chapter 3 would be an ideal diagnostic option for this. Heifers should also be tested prior to returning to the main herd, as this was a significant risk factor for lungworm disease. They could then be vaccinated if

lungworm titres were low or monitored closely and selectively treated if antibody levels were high.

However, no significant effects were seen for risk factors which were found to be significant in other studies, such as stocking rate (Schunn et al., 2013), turnout date for adult cattle (T. Schnieder et al., 1993) and the frequent purchase of cattle (Charlier et al., 2016a). There were several limitations to the cross – sectional study, particularly that the study only ran over one grazing season and participation was too low to investigate regional differences in risk factors such as anthelmintic use. It is possible that the influence of farm – based risk factors is confounded by the climate – driven infection pressure in any particular year. In essence, farm management factors may not play a significant role in a year with low or moderate infection pressure. However, if the climatic conditions are such that infection pressures are high on pasture, the influence of factors such as anthelmintic use, grazing season length and herd size may play a more significant role. The degree to which various farm management factors can protect or contribute towards increased infection pressures under climate change situations is unknown. A fascinating further study would be to combine the free – living model provided in Chapter 5 with a *D.viviparus* parasitic model, for example, the one produced by Ploeger and Eysker (2000). Farm – management practices could then be applied to this full – cycle model to investigate and design sustainable lungworm control practices under global changes such as climate change, increased environmental and economic targets for livestock farmers and the emergence of anthelmintic resistance.

The 10 – heifer pooled – milk test described in Chapter 3 had a sensitivity and specificity of 66.7% and 95.5% respectively. Although the sensitivity of the test is lower than ideal, the test performance exceeded that of testing the commonly used bulk tank samples in this study

(sensitivity 37.5% and specificity 63.6%). Moreover, the pooled – milk test presents a cheap and efficient diagnostic option. The test would be ideally suited to test herds at the end of each grazing season to plan lungworm control strategies for the following year, for example, suggesting which herds should be vaccinated. The LUNGWORM-FL model had an excellent ability to predict when infection pressures on pasture were increasing (Chapter 5). Further studies could concentrate on developing a sustainable control protocol consisting of using the pooled – milk test in the autumn to determine herds most at risk from disease and then monitoring predicted infection pressures on pasture using the LUNGWORM-FL output. Short acting anthelmintics either could be applied to the whole herd or selected individuals when the risk was seen to increase. Previous attempts at applying targeted selective treatments have treated animals who had seroconverted on monthly serum samples (Höglund, 2006). However, the duration between peak pasture infectivity and seroconversion has been found to be 36 to 46 days (Strube et al., 2017) whereas faecal patency occurs from 25 – 28 days post infection (Fiedor et al., 2009). Therefore, a targeted selective treatment based on seroconversion risks a significant amount of transmission occurring prior to treatment. However, if LUNGWORM-FL were to predict heightened risk periods, the most at risk animals in the herd (for example, first – lactation heifers) could be treated prior to seroconversion. This could be further explored using the full – cycle modelled previously described.

In summary, this thesis succeeds in answering all seven of the objectives set out in Chapter

1. However, in answering these aims, more questions have emerged which could be the target for future research in the area. These include:

1. To what degree could farm – management practices (such as alterations in grazing and pasture use, application of vaccine, targeted selective anthelmintic treatment) counteract the negative implications from climate change and emergence of anthelmintic resistance?
2. What is the greatest predictor of disease intensity: farm management practices or local climatic conditions?
3. What are the current and historic rates of lungworm vaccination use in different regions of Great Britain? How does this relate to the changing prevalence of disease?
4. How will the *D.viviparus* larvae adapt under climate change scenarios?
5. How will the *Pilobolus* spp. fungus adapt under climate change scenarios?
6. What factors influence a farmer’s decision to report a case to a passive surveillance system?
7. Can anthelmintics be applied in a sustainable way for lungworm control?
8. Can immunity to *D.viviparus* in cattle be developed whilst under anthelmintic treatment?

6.2 Difficulties encountered

The work presented in this thesis encompasses an ambitious breadth of work displaying a broad range of techniques, including but not limited to: farmer recruitment, sample collection, performing parasitology laboratory studies, questionnaire design and collation, comparison of multivariable linear regression and directed acyclic graph models and the production and validation of a mathematical model. Arguably, a complete thesis could have been built around the production, analysis and validation of a mathematical model. However, the decision to cover a broad range of objectives was taken after understanding the range of questions which needed answering for lungworm disease and the comparative lack of research interest in *D. viviparus* compared to some of the other parasitic diseases of livestock. As such, there is work that I would have liked to do but was limited given the strict three – year deadline for submission of the thesis. In particular, in Chapter 5, I would have liked to perform a sensitivity analysis on the model parameters to analyse how stable the model is under small changes in parameter estimates. This would have guided further laboratory studies aimed at ensuring that the most critical parameters are estimated accurately. However, this was limited due to time restraints with the project.

Further problems were encountered in model parameterisation. The initial plan was to perform laboratory studies on all of the lifecycle stages of larval development and migration. However, despite repeated attempts at sourcing larvae from various UK and European sources, larval mortality in transit was too high to permit performance of such experiments (as high as 100% in the majority of cases). This highlights the logistical issues with detecting lungworm larvae within faecal samples for epidemiological and clinical purposes. In hindsight, the addition of more laboratory studies would have come, regrettably, at the expense of some of the other work within the thesis. In addition, there are benefits to the

model in using a range of literature and laboratory studies. The ability for larvae to adapt under climatic stress has been well documented (see for example van Dijk and Morgan, 2008). In order to capture variations between parasite isolates who have existed in different meteorological climates (northern England vs southern England for example), it has been suggested that mathematical models should be built upon both laboratory and field isolates from a variety of sources (Rose Vineer et al., 2016). The use of laboratory studies and data from the literature enabled this complexity to be captured in a time consuming, efficient manner.

Further obstacles were overcome in the validation of the model. During the longitudinal study, herds were followed using antibody levels in milk samples rather than faecal analysis. This decision was taken primarily to increase the number of herds which could realistically be sampled at the same time, with the assumption that faecal analysis is more time consuming and time critical (due to the rapid mortality of larvae in dung) than milk sampling. However, this meant that pasture infectivity had to be assumed based on antibody titres, which although has been heavily tested in previous studies (Eysker et al., 1993; Strube et al., 2017), had to be assumed in the model rather than directly measured. Furthermore, the decision to use the VIDA database to validate yearly changes in disease intensity was taken out of necessity and is far from the ideal validation tool. One key aim for model development was to assess whether the lungworm intensity pressure differed between different areas of the country and therefore the degree to which it could predict subtle regional differences in disease outbreaks. Therefore, the use of a database that classifies cases on such low spatial resolution such as taking Scotland as a whole, brings into question the validity of using this for validation. Furthermore, as a passive surveillance system it is open to a degree of reporting bias, evidenced by the apparent reduction in cases from 2000 to 2014. However, it

represents a large dataset of temporal and to some degree, regional cases reported across the country. With transparency around the limitations, it could be used to some degree as a validation tool. Alternatives, such as data – mining of veterinary records, could have produced accurate spatial data on lungworm outbreaks across several years but would have been logistically and ethically complicated to obtain. Alternatively, a number of sentinel farms from across Great Britain could be recruited in a long-term cohort study monitoring the incidence of various parasitic diseases. However, data would not be immediately available and this would fail to identify historical trends. As mathematical models are becoming increasingly utilised in Veterinary Parasitology, attempts should be made to improve short and long – term validation data in order to assess the predictive power of these models.

Participant response during the questionnaire study in Chapter 4 was low, meaning that the study was underpowered. Moreover, there were too few respondents to compare risk factors between regions. Using the Tesco Sustainable Dairy Group (TSDG) helped to encourage participation through increased awareness and centralised support for the study but restricted responses to the TSDG group, a group of high performing dairy herds. These may have had a set of unique risk factors, confounded by their high production targets and so may not be representative of the whole of the dairy industry in the UK. Moreover, it heavily relied on the pooled heifer test (Chapter 3), a diagnostic test which is in the early stages of development and has not been externally validated (for example, through peer review or use in other studies). Therefore, the decision was made to not dichotomise farms into positive or negative but instead to keep heifer antibody titres as a continuous variable.

6.3 Conclusion




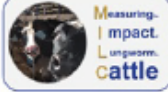
This thesis offers, for the first time, a detailed understanding of the impact that farm – management compared to climatic variables have had on producing the epidemiological changes in lungworm disease in Great Britain. It further supports claims that climate changes are already altering the parasitic disease landscape and should no longer be considered as a threat of the future. *D.viviparus* threatens to cause even more unpredictable disease under future climate change scenarios due to the altered geographical distribution and decreased seasonality of the disease. A sustainable lungworm control strategy has been suggested based on the regular monitoring of herds using the pooled – milk test and monitoring infection pressure on pasture using the LUNGWORM-FL model. Further work to integrate the free – living model with a model replicating the parasitic stages would be useful in exploring farm – management practices that can counteract the threats from climate change and increased anthelmintic resistance.

Appendix 1: Classification of Nomenclature of Territorial Units for Statistics level 2 (NUTS 2) category according to Veterinary Investigation Diagnosis Surveillance (VIDA) database

Region	Area
East	Bedfordshire Cambridgeshire Essex Hertfordshire Norfolk Suffolk
Mid and West	Derbyshire Herefordshire Leicestershire and Rutland Lincolnshire Northamptonshire Nottinghamshire Shropshire Staffordshire Warwickshire West Midlands Worcestershire
North	Cheshire Cumbria Cleveland and Darlington Durham East Riding and North Lincolnshire Greater Manchester Lancashire Merseyside Northumberland North Yorkshire South Yorkshire Tyne and Wear West Yorkshire
Scotland	Argyll and Bute Ayrshire Clyde Valley Dumfries & Galloway Eileanan an Iar East Central Fife Highlands

	Lothian North Eastern Scotland Orkney Scottish Borders Shetland Tayside
South East	Berkshire Buckinghamshire Greater London Hampshire Isle of Wight Kent Oxfordshire Surrey East Sussex West Sussex
South West	Cornwall and Isles of Scilly Devon Dorset Gloucestershire Northern Somerset and South Gloucestershire Somerset excl North Wiltshire
Wales	Carmarthenshire Ceredigion Pembrokeshire Powys North East Wales North West Wales South Wales
Unknown	Unknown
Non-GB	Channel Islands Isle of Man Northern Ireland

Appendix 2: Questionnaire used during cross – sectional study in Chapter 4.



Measuring the Impact of Lungworm in Cattle 2 (MILC 2)

Where is your farm based in the UK?

We need to know who you are so that we can find your milk samples and test them for free. We will not be sharing these details with anyone outside of the study.

2. What is your full name?

3. What is the business name for your farm?

4. What is your business address?

Address	<input type="text"/>
Address 2	<input type="text"/>
City/Town	<input type="text"/>
County	<input type="text"/>
Post Code	<input type="text"/>
Country	<input type="text"/>
Email Address	<input type="text"/>
Phone Number	<input type="text"/>

5. Which company do you milk record with?

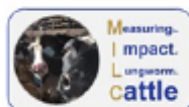
- ☐ National Milk Records (NMR)
- ☐ Cattle Information Service (CIS)
- ☐ We don't milk record

Other (please specify)

6. What is your unique NMR or CIS producer number?

7. Do you want to find out the results from the study? We can provide a report explaining your farm's results. Do you want to receive this report?

- ☐ Yes
- ☐ No



Measuring the Impact of Lungworm in Cattle 2 (MILC 2)

General farm questions

We want to ask you some questions about the general management of your farm. Please answer as truthfully as you can. There are no right or wrong answers.

8. How many animals are in your dairy herd this year?

Calves

Heifers

Adult cows

9. Do you own any beef animals on your farm?

☐ No

☐ Yes (how many?)

10. How do you replace cattle on the farm? *Please tick all that apply*

☐ Buy in cattle

☐ Breed own replacements

☐ Both

11. How many dairy cattle did you buy in last year?

Calves

Heifers

Adult cows

12. When do your cattle calve? *Please tick all that apply*

- ☐ Spring
- ☐ Autumn
- ☐ All year round

13. Have you ever been classified as an organic herd?

- ☐ No
- ☐ Yes, we still are
- ☐ Yes, but not anymore. When did you stop being classed as organic?

14. What date will you be turning out your cattle this year? *If your cattle have already been turned out, please state what date this occurred.*

	DD	MM	YYYY
Calves	<input type="text"/>	<input type="text"/>	<input type="text"/>
Heifers	<input type="text"/>	<input type="text"/>	<input type="text"/>
Adult cows	<input type="text"/>	<input type="text"/>	<input type="text"/>
Dry cows	<input type="text"/>	<input type="text"/>	<input type="text"/>

15. What date do you plan on housing your cattle this year?

	DD	MM	YYYY
Calves	<input type="text"/>	<input type="text"/>	<input type="text"/>
Heifers	<input type="text"/>	<input type="text"/>	<input type="text"/>
Adult cows	<input type="text"/>	<input type="text"/>	<input type="text"/>
Dry cows	<input type="text"/>	<input type="text"/>	<input type="text"/>



Measuring the Impact of Lungworm in Cattle 2 (MILC 2)

Contact with different animals

16. Do your cattle have any contact with another farm's cattle? *Please tick*

- ☐ No contact
- ☐ Yes, through a shared fence
- ☐ Yes, through shared grazing
- ☐ Other (please specify)

17. Do your cattle graze on land which contains a permanent water body (pond, stream, river etc) for parts of the year?

- ☐ Yes
- ☐ No

18. We want to work out whether one age group of cattle graze pasture which has been used by a different age group during the same year. Please tell us whether the following are true or false

	True	False
My calves graze pasture which was previously used by the heifers within the past 2 months	<input type="radio"/>	<input type="radio"/>
My calves graze pasture which was previously used by the adult cattle within the past 2 months	<input type="radio"/>	<input type="radio"/>
My heifers graze pasture which was previously used by the calves within the past 2 months	<input type="radio"/>	<input type="radio"/>
My heifers graze pasture which was previously used by the adult cattle within the past 2 months	<input type="radio"/>	<input type="radio"/>
My adult cattle graze pasture which was previously used by the calves within the past 2 months	<input type="radio"/>	<input type="radio"/>
My adult cattle graze pasture which was previously used by the heifers within the past 2 months	<input type="radio"/>	<input type="radio"/>
Each age group has its own pasture and pastures are rested for at least 2 months between different age groups	<input type="radio"/>	<input type="radio"/>

19. Do you graze any cattle away from your business address? *This includes groups of cattle on pasture away from the main farm for parts of the year*

- ☐ No
- ☐ Yes. Please specify what age of cattle



Measuring the Impact of Lungworm in Cattle 2 (MILC 2)

Lungworm questions

We want to ask you a few questions about lungworm (husk). There are no right or wrong answers and all answers are confidential so please answer honestly.

20. We want to know how often you see PERSISTENT COUGHING in different groups of animals. Please don't tell us about occasional, one-off bouts of coughing but list anything which is persistent or seems to have happened quite a few times. Tick all that are true from the following statements. I saw persistent coughing in.....

	Dairy Calves	Dairy Heifers	Adult dairy cows	Beef animals
This year	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Last year	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2 years ago	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3 - 5 years ago	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Longer than 5 years ago	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Never	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Don't know	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

21. Now we want to think about more serious lungworm outbreaks. This could include times when you saw a significant milk drop, times when several animals were clinically ill or even times when cattle have died due to lungworm.

I have had a serious problem with lungworm.....(tick all that apply)

- ☐ This year
- ☐ Last year
- ☐ 2 years ago
- ☐ 3-5 years ago
- ☐ Longer than 5 years ago
- ☐ Never
- ☐ Don't know

22. Has your vet ever diagnosed lungworm on the farm?

- ☐ Yes
- ☐ No
- ☐ Don't know

23. How was lungworm diagnosed by your vet and when was this done?

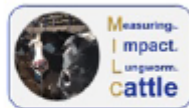
	Dung test	Blood test	Individual milk test	Bulk tank milk test	Post mortem	I don't know what test my vet used
This year	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Last year	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2 years ago	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3 - 5 years ago	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Longer than 5 years ago	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
My vet has not diagnosed lungworm with this method	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Other (please specify)

24. Do you think you have lungworm on your farm?

- ☐ Yes
- ☐ No
- ☐ Don't know

25. Why do you think that you do or don't have lungworm on your farm?



Measuring the Impact of Lungworm in Cattle 2 (MILC 2)

Lungworm vaccine

We want to ask you some questions about the lungworm vaccine (Huskvac). Please answer truthfully. There are no right or wrong answers.

26. Do you use the lungworm vaccine, Huskvac?

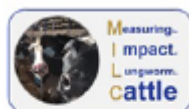
- ☐ Yes
- ☐ No
- ☐ Don't know
- ☐ Why do you or don't you use Huskvac?

27. How soon after giving the SECOND DOSE OF THE vaccine do you turn the vaccinated cattle out?

- ☐ 1 week later
- ☐ 2 weeks later
- ☐ 1 month later
- ☐ 2 months later
- ☐ Longer than 2 months
- ☐ Already turned out when I vaccinate them

28. Please tell us when you use Huskvac in different ages of cattle. *Please tick all that apply.*

	Newborn calves	First season grazers	Second season grazers	Milking cattle
January	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
February	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
March	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
April	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
May	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
June	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
July	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
August	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
September	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
October	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
November	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
December	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
I don't give Huskvac to this age group of cattle	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>



Measuring the Impact of Lungworm in Cattle 2 (MILC 2)

Worming your cattle

Finally, we want to ask you some questions about what type of wormers you use in your cattle. Again, please answer honestly. There are no right or wrong answers.

29. We want to understand whether you gave your cattle worming products from **April 2016 to September 2016**. Please tick the box if you gave worming products to each group of cattle:

	April 2016	May 2016	June 2016	July 2016	August 2016	Septmeber 2016
Calves	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Heifers	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Dry cows	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Adult milking cows	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

30. Please tell us the name of the wormers you gave to your cattle from **April 2016 to September 2016**:

Calves	<input type="text"/>
Heifers	<input type="text"/>
Dry cows	<input type="text"/>
Adult milking cows	<input type="text"/>

31. Now consider the later part of the year. From **October 2016 to March 2017**, please tick the box if you gave wormers to any group of cattle:

	October 2016	November 2016	December 2016	January 2016	February 2016	March 2016
Calves	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Heifers	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Dry cows	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Adult milking cows	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

32. Please tell us the name of the wormers you gave to your cattle from **October 2016 to March 2017**:

Calves	<input type="text"/>
Heifers	<input type="text"/>
Dry cows	<input type="text"/>
Adult milking cows	<input type="text"/>

33. Do you quarantine bought-in animals as they enter your farm?

- ☐ No
- ☐ Yes, for how long?

34. Do you give any wormers to your cattle as they are bought in as part of protocol?

- ☐ No
- ☐ Yes, Please write the name of the product

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